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THE RELATION OF SUMATRAN MITE FEVER TO THE TSUTSUGAMUSHI DISEASE OF BRITISH MALAYA.

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EARLIER investigations (1936*a*) on the relation between so-called scrub-typhus (*syn.* rural typhus) and the tsutsugamushi disease of Malaya have convinced us that the distinction between the two, a clinical one, is no longer valid; that in both a dermal lesion varying in degree of development always occurs, although it may have disappeared before the patient seeks medical aid. Scrub-typhus is not an entity and must disappear from the nomenclature. Only one typhus-like disease of the XK serological type exists in British Malaya, *viz.* tsutsugamushi.

In the neighbouring Netherlands Indies, especially in Sumatra, a similar problem of classification exists concerning the pseudo-typhus of Schüffner, now more usually called Sumatran mite-fever, a typhus-like disease of the XK serological type that is marked clinically by the presence of a primary ulcer. Kouwenaar and Wolff (1936) take the view that mite-fever differs from tsutsugamushi and more nearly resembles scrub-typhus; that it is scrub-typhus with an additional clinical sign, *viz.* a primary ulcer.

Manifestly confusion exists, and in 1938 we exchanged strains with our Dutch co-workers with a view to carrying out cross-immunity tests, these being the best available means for comparing strains of the typhus-like fevers. The virus of the Seerangayee strain of tsutsugamushi was sent from this laboratory by air to Sumatra as infected peritoneal exudate from a passage guinea-pig. Insomuch as this strain was originally isolated (in 1931) from a patient who showed the typical syndrome of tsutsugamushi except that no primary lesion could be found, it was until recently designated scrub-typhus. In return we received from Dr. Kouwenaar and Dr. Wolff strains of Sumatran mite-fever in white mice. (We wish to thank them for placing the strains at our disposal.)

EXPERIMENTAL MITE-FEVER.

In the White Mouse.

Two strains were received, each in three white mice. Contrary to the experience of the Dutch workers, we were unable to maintain either strain in white mice for long, each dying out by the third generation of passage.

In the Guinea-pig.

By intra-peritoneal inoculation of brain or peritoneal exudate from the white mice sent to us, we tried without success to infect guinea-pigs that had been fed on a vitamin-deficient diet. This result was not unexpected, since the guinea-pig is not the animal of choice for the XK type of strains.

In the Rabbit.

Use of this animal was immediately successful. By the intra-ocular inoculation of rabbits with peritoneal exudate from the infected white mice received, strains were established from four of the mice. One of them has been maintained in rabbits for two years by successive intra-ocular passage of infected aqueous humour and has passed through more than 70 generations without loss of virulence.

The ocular signs of infection have been identical with those seen in experimental tsutsugamushi disease. The strain has, however, been less virulent than the stock strain of tsutsugamushi that has been maintained in this laboratory since its isolation from a human case occurring locally eight years ago; the incubation period and the development of the iridocyclitis have been longer, and in a considerable minority of rabbits, the intensity of the reaction has been less. Smears of the endothelium covering Descemet's membrane, when stained by Giemsa's method, reveal abundant intra-cellular *Rickettsia* indistinguishable in staining characteristics, morphology and distribution, from *R. tsutsugamushi* (syn. *R. orientalis*), the causal organism of the tsutsugamushi disease (*v.* previous publications from this Institute).

Successive Weil-Felix reactions (for the results of which we are indebted to Dr. R. Green, Senior Bacteriologist to this Institute) were done on ten of the rabbits. Four rabbits gave positive results, the maximum titres in each case being respectively 1/250 OXK (twice), 1/110 OXK, and 1/96 OXK on the 42nd, 46th, 47th and 18th day after inoculation.

In the Monkey.

One gibbon and eight macacus monkeys have been inoculated intradermally. The results agree closely with those recorded by Kouwenaar and Wolff (1934). In the gibbon: (1) There was no febrile reaction. (2) Leucopenia was severe. (3) At each of the three sites of inoculation there developed a papule, in one of which marked central necrosis occurred. (4) The Weil-Felix reaction was positive, a maximum titre of 1/340 OXK being attained on the 21st day. In the eight macacus monkeys: (1) A febrile reaction was absent in three cases; it was mild in the other five, its duration being 8, 6, 3, 3, and 2 days respectively. (2) Leucopenia was slight in one, mild in two, severe in five.

(3) Dermal lesions were absent in two cases, but present in the other six as papules; in three cases these were small and fleeting, in the other three they were well marked, persistent and developed central necrosis. (4) The Weil-Felix reactions in the macacus monkeys were positive in all eight; only the OXK strains of *Proteus* were agglutinated, the maximum titres being respectively 1/85, 1/125 (in three), 1/170, 1/220 (in two), and 1/250.

There are thus four criteria of infection, *viz.* fever, leucopenia, dermal lesions and development of a positive Weil-Felix reaction OXK in type.

The brevity of the febrile reaction, the occasional absence of fever, the differing degree of dermal reaction, and the range of maximum Weil-Felix titres attained in the macacus monkeys agree closely with the corresponding findings noted by us (1936*b*) in infections of monkeys by the intradermal route with the Raub and Seerangayee strains of tsutsugamushi.

CROSS-IMMUNITY TESTS.

Cross-immunity tests were done in rabbits and monkeys.

In Rabbits.

For comparison with the strain of mite-fever, three strains of the XK type were used. They were:

(*a*) The stock Raub strain of tsutsugamushi, derived from a patient with a well-marked ulcer on admission to hospital.

(*b*) The stock Seerangayee strain of tsutsugamushi, derived from a patient in whom no ulcer could be found. This strain is immunologically identical with the Raub strain. Usually maintained in the guinea-pig, it was transferred to the rabbit by the intra-ocular inoculation of virus for the purposes of this investigation.

(*c*) A strain of E.K. 2 originally isolated from a wild rat, which, after passage through fleas, behaved in both guinea-pig and rabbit as an XK type of strain; it showed, especially, immunological identity with both the Raub and the Seerangayee strains.

In the interpretation of the ocular reactions obtained during an investigation of cross-immunity of typhus-like strains of XK type, it is necessary to bear in mind that, as we have observed (1936*a*), the inoculation of a virus into the unused eye of a rabbit immune to that particular virus will, in a small minority of cases, result in insignificant and fleeting ocular signs of two to three days' duration only. Such transitory reactions contrast markedly with the intensity and duration of the reaction in a non-immune animal, and they are not considered of any significance in the assessment of the relationship of one strain to another. We obtained similar reactions in cross-protection tests between five strains of tsutsugamushi isolated from five patients who had contracted the disease within a few months of each other in the same endemic area.

The term "partial immunity" that is used below signifies an ocular reaction that in intensity and duration exceeded the above described transitory reactions, yet in these features fell far short of the complete reaction seen in passage animals, or obtained when a rabbit immune to an XK type of virus is reinoculated with a virus of X19 type (or *vice versa*).

A control rabbit was inoculated and reacted typically in every case.

Mite-fever virus as inoculum.

(i) Seven convalescent rabbits of the Raub strain of tsutsugamushi were reinoculated into the unused eye with the virus of mite-fever ; six showed no trace of reaction, *i.e.* were immune ; one showed a mild reaction that signified partial immunity.

(ii) Two convalescent rabbits of the Seerangayee strain of tsutsugamushi were reinoculated with mite-fever virus ; both proved to be immune.

(iii) Two convalescent rabbits of the E.K. 2 strain were similarly reinoculated ; both proved to be immune.

Thus, of eleven rabbits convalescent to stock strains of the XK type, ten showed complete immunity, one a partial immunity.

Stock strains of XK type as inoculum.

(i) Sixteen convalescent rabbits of the mite-fever strain were reinoculated with the virus of the Raub strain of tsutsugamushi. Two only proved to be completely immune ; ten showed a partial immunity ; four showed a trace of immunity, the reaction in these four being a little less intense and the waning of the reaction more rapid than in the reaction of the control rabbit.

(ii) Three convalescent rabbits of the mite-fever strain were reinoculated with the virus of the Seerangayee strain of tsutsugamushi ; two proved to be completely immune ; one showed partial immunity.

(iii) Two convalescent rabbits of the mite-fever strain were reinoculated with virus of the strain E.K. 2 ; both proved to be completely immune.

Thus, of 21 rabbits convalescent from infection with the mite-fever strain, six proved to be completely immune, eleven to be partially immune, and four to have only a slight degree of immunity, to virus of XK type. There was thus an incomplete " one-way " cross-immunity, in contrast to the almost complete cross-immunity found in the converse experiments described earlier.

It will be noted, however, that ten of eleven rabbits that showed a partial immunity and four that showed only a slight immunity were convalescents that were reinoculated with the Raub strain. This Raub strain is more virulent than the other strains ; the incubation period is much shorter, the evolution of the ocular signs more rapid and the maximum intensity of the reaction greater. Some years ago we had occasion to compare with the Raub strain another strain (McNab) of tsutsugamushi isolated from a patient under our care, whose clinical syndrome included the presence of a primary ulcer. The same greater virulence of the Raub strain obtained. The cross-immunity results resembled broadly those described above, in that convalescent rabbits of the Raub tsutsugamushi strain did not react to reinoculation of the McNab strain, whereas four or five convalescent rabbits of the latter strain gave mild reactions to reinoculation by the Raub strain. Those reactions were, indeed, milder than the eleven partial reactions observed in the present mite-fever investigation. Their occurrence in similar circumstances, however, *viz.* the inoculation of a more virulent virus into rabbits immune to a milder virus, and their rarity in the converse experiments, suggest strongly that the partial reactions of the present investigation caused by the inoculation of the Raub strain into rabbits immunized against mite-fever were due to the differing

virulence of these two strains in the rabbit. Directly comparable results have been recorded in the Rocky Mountain spotted-fever group of typhus fevers. Reimann *et al.* (1932) isolated a typhus-like strain (" Minnesota ") that immunological and pathological study has placed in the spotted fever group. It was, however, of low virulence as compared with other strains of this group. Although these other strains immunize against it, it conferred only an irregular immunity against them. Commenting on these findings Pinkerton (1936) states: " In cross-immunity tests between two strains of a disease which are essentially identical, but which vary markedly in virulence, we may expect the more virulent one always to protect completely against the less virulent one, but are not surprised if the protection afforded by the less virulent one against the more virulent one is incomplete." By morphological studies of the causal organism in smears, sections and tissue cultures, he was able to confirm the identity of the " Minnesota " strain with the spotted fever group. Zinsser (1937), discussing the classification of the " Minnesota " strain, concludes: " Its peculiarities can be more logically attributed to relatively low virulence than to qualitative difference."

In Monkeys.

For comparison with the strain of mite-fever, the Raub and the Seerangayee strains of tsutsugamushi were used.

All inoculations of virus were made by the intradermal route.

In a preliminary experiment a macacus monkey, that had reacted to an inoculation of mite-fever virus with fever, leucopenia, dermal lesions, and a positive Weil-Felix reaction, was inoculated nine weeks later with the same virus (Table I).

TABLE I.*

Animal.	Nature of infection.	Weeks.	Criteria of infection. F°.	L.	D.L.	Weil-Felix.
Macacus 18	Original	—	8	+	P.CN	1/250
	Test	9	0	0	0	1/110
Gibbon (control)		—	0	++	P.CN	1/340

* The figures given in this and other tables for the Weil-Felix reaction were the maximum titres attained. They refer to the " OXK " strain of *Proteus*; significant agglutination of the " OX19 " strain never occurred. Serum was obtained before inoculation, and subsequently at intervals of five days from the 12th day onwards for 60 days. Leucocyte counts were made before inoculation, and later every five days from the seventh day onwards until the figures returned to the normal level.

The results of the original inoculation, by which the monkey became immune, are given first in each test. A control monkey was inoculated in each test.

The abbreviations used in this and other tables are as follows: Weeks = number of weeks elapsed between original and test inoculation. F° = duration in days of febrile reaction. L = Leucopenia. D.L. = Dermal lesions. P = Papule. CN = Central necrosis.

± = Diminution of leucocytes by less than one-quarter.
\+ = " " one-quarter.
+± = " " one-half.
++ = " " more than one-half.
O = No reaction.

The results in Table I show that the test monkey gave only one of the four criteria of infection, *viz.* a positive Weil-Felix reaction, which in this case was of low titre and very transient, whereas the control gave three criteria.

Mite-fever strain as inoculum.

One gibbon and two macacus monkeys that had recovered from an experimental infection with the Raub strain of tsutsugamushi were reinoculated with the virus of the mite fever strain (Table II).

TABLE II.

Number of test.	Animal.	Nature of infection.	Weeks.	Criteria of infection. F°.	L.	D.L.	Weil-Felix.
1	Gibbon 1*	Original.	—	6	++	P	1/3850
				(0)	(±)	(P)	(0)
		Test	8	0	±	0	0
	Macacus (control)		—	0	++	P	1/125
2	Macacus 8	Original.	—	0	+	P.CN	1/340
		Test	15	0	0	0	0
	Macacus (control)		—	2	±	P.CN	1/220
3	Macacus 14	Original.	—	3	±	P.CN	1/190
		Test	15	0	0	0	0
	Macacus (control)		—	3	0	P.CN	1/220

* Gibbon originally inoculated with tsutsugamushi virus (results unbracketed) four years ago; reinoculated with the same virus eight weeks prior to this cross-protection test (results in brackets) to ensure its immunity to tsutsugamushi.

Four macacus monkeys that had recovered from an experimental infection with the Seerangayee strain of tsutsugamushi were reinoculated with the mite-fever strain (Table III).

TABLE III.

Number of test.	Animal.	Nature of infection.	Weeks.	Criteria of infection. F°.	L.	D.L.	Weil-Felix.
1	Macacus 11	Original.	—	0	±	P	1/96
		Test	13	0	0	0	0
	Macacus (control)		—	8	+	P.CN	1/250
2	Macacus 12	Original.	—	0	++	P	0
		Test	13	0	0	0	1/170
	Macacus (control)		—	0	±	P	1/170
3	Macacus 18	Original.	—	0	+	P.CN	1/125
		Test	14	0	0	0	0
	Macacus (control)		—	3	0	P.CN	1/220
4	Macacus 19	Original.	—	0	±	P.CN	1/192
		Test	8	0	0	0	0
	Macacus (control)		—	6	+	0	1/125

To summarize the seven cross-protection tests of Tables II and III : five test monkeys showed no reaction, one yielded only a leucopenia, another only a mildly positive Weil-Felix reaction, whereas the seven control monkeys reacted strongly.

Stock Strains of XK type as inoculum.

One gibbon and two macacus monkeys that had recovered from an experimental infection with the virus of mite-fever were reinoculated with virus of the Raub strain of tsutsugamushi (Table IV).

TABLE IV.

Number of test.	Animal.	Nature of infection.	Weeks.	Criteria of infection. F°.	L.	D.L.	Weil-Felix.
1	Gibbon III	Original.	—	0	+±	P.CN	1/340
		Test	9	0	+	0	1/125
	Macacus (control)		—	0	+	P.CN	1/770
2	Macacus 9	Original.	—	3	+±	0	1/125
		Test	11	0	0	0	0
	Macacus (control)		—	5	++	P.CN	1/190
3	Macacus 18	Original.	—	8	+	P.CN	1/250
		Test	18	0	0	0	1/770
	Macacus (control)		—	0	+	P.CN	1/770

Three macacus monkeys that had recovered from an experimental infection with the mite-fever strain were reinoculated with virus of the Seerangayee strain of tsutsugamushi (Table V).

TABLE V.

Number of test.	Animal.	Nature of infection.	Weeks.	Criteria of infection. F°.	L.	D.L.	Weil-Felix.
1	Macacus 16	Original.	—	2	+±	P.CN	1/220
		Test	8	0	0	P.CN	0
	Macacus (control)		—	0	+±	P.CN	1/192
2	Macacus 17	Original.	—	0	+±	P	1/170
		Test	6	0	0	0	0
	Macacus (control)		—	0	+±	P.CN	0
3	Macacus 21	Original.	—	6	+	0	1/125
		Test	17	0	0	0	1/110
	Macacus (control)		—	0	+	P	1/125

To summarize the findings in Tables IV and V : two test monkeys showed none of the criteria of infection ; three, one criterion only ; one, two criteria. In contrast, the control monkeys reacted strongly.

DISCUSSION.

Reviewing the results in rabbits and monkeys, the most arresting one was that obtained when rabbits immune to mite-fever were reinoculated with virus of the Raub strain of tsutsugamushi, in that 14 out of 16 proved to be only partially immune. As stated above, we consider that this finding loses its apparent import when the difference in the degree of virulence in the rabbit, between the two strains, is taken into account. Moreover, similar cross-immunity tests in the rabbit with the Seerangayee and E.K. 2 strains, both of a degree of virulence similar to that of the mite-fever strain, showed that four of five convalescents of the mite-fever strain were completely immune. In monkeys the findings consistently indicate a close similarity in the immunogenic properties of the mite-fever and tsutsugamushi strains.

In our view the results of the cross-immunity test considered comprehensively indicate that the strain of Sumatran mite-fever and the three tsutsugamushi strains compared with it are strains of one and the same disease. Such differing immunogenic features as they show may well be found between different strains of any infectious disease. The close similarity of mite-fever and tsutsugamushi in aetiology, epidemiology, clinical syndrome in man, serology and experimental pathology is thus found also in their immunogenic properties.

One further point calls for comment. Kouwenaar and Wolff (1935) regard Sumatran mite-fever as distinct from the " classical " tsutsugamushi of Japan. They base this distinction on experiments which they made on three Japanese monkeys (*Macacus fuscatus*). These they obtained from Japan, and inoculated them with the virus of mite-fever. Although these monkeys reacted as did the local monkeys, they showed quantitative differences. Moreover, one monkey that had been experimentally infected in Japan with the virus of tsutsugamushi and later proved to be immune there was reinoculated by them with the virus of mite-fever on its arrival in Sumatra two-and-a-half years later ; it reacted typically and completely.

That quantitative differences occur in the reaction of monkeys to tsutsugamushi strains of Malaya is amply shown earlier in this paper ; not each of the four criteria of infection may develop, and differences in degree of reaction are common.

Concerning the degree of immunity conferred in monkeys, in our experience immunity to the experimental tsutsugamushi disease of Malaya is not of long duration. Thus a gibbon (Gibbon 1, Table II) that had reacted with all four criteria of infection to the virus of the tsutsugamushi of Malaya was reinoculated by us with the homologous virus four years later ; two criteria of infection, *viz.* marked leucopenia and papule formation, developed. Another gibbon that had reacted to the Raub strain of tsutsugamushi with three criteria of infection was reinoculated by us 15 months later with the homologous virus ; a characteristic ulcer and a moderate leucopenia developed. Another gibbon convalescent from experimental tsutsugamushi infection was reinoculated two months later ; one criterion of infection, a leucopenia, again developed. Moreover, Prof. Norio Ogata (personal communication) is of opinion that the duration of the immunity in monkeys inoculated with the " classical " tsutsugamushi of Japan may be as short as three months. It is therefore difficult

to resist the conclusion that the marked reaction observed by the Dutch workers on inoculating the virus of mite-fever into the monkey which proved immune in Japan two and a half years earlier, might not have been due to that early fading of immunity that we in Malaya have noted, and which they (Kouwenaar and Wolff, 1934) noted in monkeys from British India and Sumatra.

SUMMARY.

1. Cross-protection tests between a strain of Sumatran mite-fever and strains of the tsutsugamushi disease were done in the rabbit and monkey with the object of elucidating the inter-relation of these two diseases.

2. It is concluded that Sumatran mite-fever is not a disease *sui generis*, but is identical with the tsutsugamushi disease.

REFERENCES.

KOUWENAAR, W., AND WOLFF, J. W.—(1934) *J. infect. Dis.*, **55**, 315.—(1935) *Geneesk. Tijdschr. Ned. Ind.*, **75**, 34.—(1936) *Zbl. Bakt.*, I Abt., Orig., **135**, 427.

LEWTHWAITE, R., AND SAVOOR, S. R.—(1936*a*) *Brit. J. exp. Path.*, **17**, 448.—(1936*b*) *Ibid.*, **17**, 1.

PINKERTON, H.—(1936) *Parasitology*, **28**, 172.

REIMANN, H. A., ULRICH, H. L., AND FISHER, L. C.—(1932) *J. Amer. med. Ass.*, **98**, 1875.

ZINSSER, H.—(1937) *Amer. J. Hyg.*, **25**, 430.

THE EFFECT OF LYSOZYME ON THE UNION BETWEEN A PHAGE AND THE SUSCEPTIBLE *BACILLUS MEGATHERIUM*.

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WOLLMAN and Wollman (1936) reported that addition of egg-white lysozyme to a lysogenic strain of *B. megatherium* released phage. This seemed to be a specific effect, for grinding up the bacteria with glass beads or treating them with trypsin did not increase the phage titre. Gratia (1936) could not repeat these results using the same strain of *B. megatherium*, but Bruce White (1937) found a similar effect using a weakly lysogenic strain of *V. cholerae*. The chemical mechanisms of phage adsorption, multiplication and release are little understood, and it seemed possible that a further study of the effect of lysozyme on the union of phage with the bacterium might be of interest. Lysozyme has been shown by Meyer *et al.* (1936) to be a carbohydrase which hydrolyses the polysaccharide moiety of egg and bacterial mucins. Phage is thought to be primarily attached to the specific polysaccharide of the bacterial surface, therefore the action of lysozyme might be due to the hydrolysis of this polysaccharide with subsequent release of phage.

In this study the same strains of *B. megatherium* were used as in Wollman's

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(1936) work. Phage was obtained from a lysogenic culture of *B. megatherium* L and was titrated on the sensitive strain *B. megatherium* M. Exact repetition of Wollman's results was not attempted, but the effect of lysozyme on the union of phage with heat-killed organisms has been studied. Dilute egg-white has been used as the source of lysozyme. It is known that many phages are specifically adsorbed on to heat-killed sensitive bacteria and can be removed from a suspension in this way.

EXPERIMENTAL METHODS.

Preparation of phage.

B. megatherium L was grown overnight in Roux bottles in a thin layer of 2 per cent. Difco proteose peptone. The culture was centrifuged to remove the organisms and the supernatant was filtered through an L2 candle. The filtrate usually had a titre of 10^4–10^6 phage particles per ml.

Cultivation of B. megatherium M.

B. megatherium M was grown on nutrient agar made with a tryptic digest of casein. In preparing heated organisms an overnight culture was washed off the medium with water and the suspension quickly heated to 80–85° C. in a water bath. It was maintained at this temperature for half an hour and then centrifuged. The organisms were re-suspended in a small volume of water and stored at 0° C.

Titration of phage.—Phage titre was estimated by the plaque method. The counting method was a modification of the surface viable count of Miles and Misra (1938), in which the drop areas are of phage rather than of bacteria. The results recorded are the means of plaque counts in 6–12 drop areas, reduced to the nearest integer. Calculations of the statistical significance of the differences between the counts are unnecessary since differences of less than a factor of 10 are not considered relevant. Plaque counts were made after 6–8 hours' growth for, if the plates were left overnight, the bacteria sometimes obscured the plaques. The most regular results were obtained if the culture of *B. megatherium* M, on which phage was to be titrated, was grown on an agar slope overnight at 37° C. The growth was then suspended in 2–3 ml. of broth, and about 1 ml. was pipetted on to the surface of agar plates that had been dried for 24 hours at 37° C. The bacterial suspension was spread over the plate by tilting and turning. After 2–3 mins. the excess fluid was pipetted off and the plates left on the bench for 10 mins. to soak. They were then put open in the incubator at 37° C. for about 1½ hours. In this way an even growth of *B. megatherium* M was usually obtained. If the growth was not even it was spread with a sterile glass rod immediately before the titration of phage was carried out.

Estimation of carbohydrates.

Reducing sugars.—These were estimated by Giragossintz, Davidson and Kirk's (1936) method. The ferrocyanide formed was titrated with cerium sulphate in acid solution; 0·2 per cent. Xylene cyanol FF was used as an indicator. The mixtures were de-proteinized with cadmium, Fujita and

Iwatake (1931); 3·5 ml. $CdSO_4$ (13 g. per l. of 0·06 *N* H_2SO_4) and 0·75 ml. of 0·62 *N* NaOH were added to 1·0 ml. of the fluid under investigation. The precipitate was centrifuged off and 2·0 ml. of the clear supernatant was used for each estimation. Duplicates of the samples and controls of the reagents were always done. One ml. of the cerium sulphate used was equivalent to 0·1 mg. glucose.

Total carbohydrate.—The orcin colorimetric method described by Pirie (1936) was used.

RESULTS.

Tables I and II give the results of experiments showing the release of phage from heat-killed organisms by lysozyme; pangestin and takadiastase had no effect. Pangestin does not injure phage, since phage is released from phage-coated bacteria by a mixture of pangestin and lysozyme to the same extent as by lysozyme alone. Unlike lysozyme, however, pangestin digests heat-killed bacteria; this is evidence that it is not simply lysis of the bacterium that releases phage.

TABLE I.—*Release of Phage from Heat-killed* B. megatherium *M by Dilute Egg-white.*

Exp. number.	Phage particles/ml. supernatant after treatment with—	
	Saline.	Lysozyme.
1	5×10^4	1×10^6
2	3×10^4	1×10^6
3	3×10^3	1×10^7
4	4×10^3	2×10^6
5	2×10^2	4×10^4

TABLE II.—*Comparison of the Effect of Pangestin, Takadiastase and Lysozyme on the Union between Phage and Heat-killed* B. megatherium *M.*

(*a*) Titres of supernatants from heated, phage-coated *B. megatherium* M:

Exp. number.	Phage particles/ml. supernatant after treatment with—				
	Saline.	Lysozyme.	Pangestin.	Pangestin + lysozyme.	Takadiastase.
1	1×10^6	$>1 \times 10^7$	3×10^5	$>1 \times 10^7$	..
2	1×10^3	2×10^5	1×10^3	..	2×10^3

(*b*) Weight of remaining undissolved bacteria:

Exp. number.	μg. bacteria/ml. suspension after treatment with—				
	Saline.	Lysozyme.	Pangestin.	Pangestin + hysozyme.	Takadiastase.
1	185	175	66	15	..
2	162	132	37	..	143

Table I shows the release of phage from heat-killed *B. megatherium* M by dilute egg-white. One ml. heat-killed *B. megatherium* M (containing about 0·8 mg. dry wt. of bacteria per ml.) and 5·0 ml. phage suspension were left

at 0° C. for 15 mins. and then centrifuged to sediment the bacteria. Five ml. more phage were added to the sediment, which was re-suspended and again left at 0° C. for 15 mins. This process was repeated in all three or four times. After the final centrifugation the sediment was washed on the centrifuge with phosphate buffer of pH 7·2. It was then re-suspended in 4·5 ml. of buffer and 1·0 ml. was put in each of four conical centrifuge tubes. The suspensions were centrifuged and the sediments were made up in 1·0 ml. of saline or of solutions of enzymes in saline ; 0·2 per cent. egg-white (0·03 per cent. on basis of dry weight) was used. The mixtures were left overnight at 0° C. and then for 1 hour at 37° C. They were then centrifuged and the supernatants were titrated for phage.

In Table II the effect of pangestin, takadiastase and lysozyme on the union between phage and heat-killed *B. megatherium* M are compared. The set-up was the same as for the experiments of Table I. At the end of the experiments the sediments were re-suspended in their original volumes and the weight of undissolved bacteria remaining was determined by a turbidity method described by Clifton, Mueller and Rogers (1935).

Heated *B. megatherium* M which had been incubated with egg-white could no longer adsorb phage. A suspension of heated bacteria was prepared in the usual way and divided into two parts. Egg-white to a final concentration of 1/500 was added to one part and then both were incubated at 37° C. for several days in the presence of toluene. Both suspensions were then heated to 85° C. for ½ hour to destroy lysozyme. The bacteria were then centrifuged out and made up in a small volume of water, frozen and dried. Equal weights of these two preparations were suspended in water and tested for their power to adsorb phage. A phage suspension was diluted to give 50–100 plaques per drop and an experiment set up as shown in Table III. Nearly all the phage was removed from the suspension by the heated bacteria so that few plaques were produced by the supernatant. On the other hand, the supernatant from the heated, lysozyme-treated bacteria gave as many plaques as the control showing that these bacteria had not adsorbed phage. In order to control the effect of residual lysozyme a tube (4) was set up in which phage was mixed with both heated and heated, lysozyme-treated, bacteria. The adsorption of phage here was as great as in the case where heated bacteria alone were used, showing that lysozyme, even if present in traces, was having no effect. The total carbohydrate content of the heated bacteria was between 8 and 10 per cent. After treatment with lysozyme it fell to between 2 and 3 per cent.

TABLE III.—*Adsorption of Phage by heated* B. megatherium *M before and after Treatment with Lysozyme.*

Small conical centrifuge tubes were set up as follows :

(1) 0·5 ml. phage + 0·5 ml. H_2O.
(2) 0·5 ml. phage + 0·1 ml. heated bacteria + 0·4 ml. H_2O.
(3) 0·5 ml. phage + 0·1 ml. heated, lysozyme-treated bacteria + 0·4 ml. H_2O.
(4) 0·5 ml. phage + 0·1 ml. heated bacteria + 0·1 ml. heated, lysozyme-treated bacteria + 0·3 ml. H_2O.

The tubes were left at 0° C. overnight and then on the bench for two hours. They were then centrifuged for 5 mins. at 3000 r.p.m. and the supernatants were titrated for phage. The results are expressed in number of plaques per drop. Each figure is the mean of 9–12 counts.

Exp. number.	Plaques/drop, from tube number— 1.	2.	3.	4.	Weight bacteria/ml. mixture. (mg.).
1	70	3	74	5	1·0
2	71	4	76	5	1·0
3	80	4	65	21	0·5
4	35	2	31	..	0·5
	35	..	30	..	2·0
5	66	7	48	..	0·5

This removal of carbohydrate from heated *B. megatherium* M by lysozyme was next investigated more closely in order to see whether hydrolysis of a polysaccharide with production of reducing sugars occurred as in the lysis of sarcinae by lysozyme. Table IV shows that when autolysing or heat-killed bacteria are incubated with lysozyme, reducing substances are produced. In the first case lysis of the bacteria also occurs, but in the second case there is no lysis unless alkali is added to the suspension.

TABLE IV.—*Production of Reducing Substances from* B. megatherium *M by Egg-white Lysozyme.*

Tubes were set up as follows :

	ml. in tube number— 1.	2.	3.
Bacterial substrate (*ca.* 10 mg./ml.)	3·0	3·0	..
M/2 phosphate buffer pH 7·2	0·1	0·1	0·1
Filtered 1/3 egg-white	0·1	..	0·1
Water	..	0·1	3·0

M/600 sodium iodoacetate or a drop of toluene was added to prevent bacterial growth. The tubes were incubated at 37° C. One ml. samples were taken before incubation and after various periods and the content of reducing sugars estimated.

Exp. number.	Time. (hours.)	Increase in reducing substances as μg. glucose/ml. in tube number— 1.	2.	3.	Bacterial substrate.
1	20	90	0	0	Heated *B. megatherium* M.
2	22	84	0	0	Ditto.
3	5¼	55	16	0	,,
4	3¼	263	..	0	,,
5	3¼	240	..	0	,, after digestion with pangestin.
6	3¼	322	66	0	Autolysing *B. megatherium* M.
7	5¾	64	0	..	Heated *B. megatherium* M.

TABLE V.—*Rates of* (a) *Phage Release from Heat-killed Phage-coated Bacteria, and* (b) *Hydrolysis of Carbohydrate from these Bacteria, by Dilute Egg-white.*

(a) *Phage Titres. Phage Particles/ml. Supernatant.*

Time. (mins.)	Exp. number: 1. Control.	1. Egg-white. (0·05 per cent.)	2. Control.	2. Egg-white. (0·1 per cent.)	3. Control.	3. Egg-white. (0·2 per cent.)	4. Control.	4. Egg-white. (0·2 per cent.)
0	8×10^4	8×10^4	3×10^2	4×10^2	8×10^2	8×10^2	1×10^5	1×10^5
10	..	..	..	..	..	1×10^4	..	..
15	..	1×10^6	..	7×10^3	..	..	..	..
20	..	..	..	..	..	5×10^4	..	..
30	3×10^5	1×10^6	3×10^3	7×10^3	4×10^3	2×10^5	..	3×10^6
45	..	2×10^6	..	2×10^4	..	..	..	..
60	2×10^5	4×10^6	..	4×10^4	8×10^2	1×10^5	2×10^5	2×10^6
Total change	×2·3	×50	× 10	× 100	× 0	× 120	× 2	× 20

(b) *Total carbohydrate of Bacterial Sediments.*

Time. (mins.)	Total carbohydrate (as μg glucose/ml.) Exp. number: 1. Control.	1. Egg-white. (0·05 per cent.)	2. Control.	2. Egg-white. (0·1 per cent.)	3. Control.	3. Egg-white. (0·2 per cent.)	4. Control.	4. Egg-white. (0·2 per cent.)
0	303	303	322	375	370	333	160	150
10	..	..	..	..	343	300	..	..
15	294	270	312	301	..	..	..	..
20	..	..	..	..	322	235	..	..
30	294	200	333	220	370	163	144	63
45	281	136	322	160	..	..	..	..
60	277	117	322	125	312	156	150	55
Total change	— 26	— 186	— 0	— 250	— 58	— 177	— 10	— 95

A comparison between the rates at which lysozyme released phage from heat-killed, phage-coated bacteria and hydrolysed the carbohydrate contained in them was made. Phage was adsorbed on to heat-killed *B. megatherium* M in the usual way. Equal amounts of bacteria (about 1–2 mg./ml. final concentration) were suspended in equal volumes of saline or dilute (0·2–0·05 per cent.) egg-white in saline. The mixtures were incubated at 37° C. and samples were taken at once and at stated intervals. They were cooled to 0° C. and centrifuged. The phage titre of the supernatant and the carbohydrate content of the bacterial sediment were determined. With the concentrations of bacteria and of enzyme that were used almost complete release of phage and hydrolysis of bacterial carbohydrate took place during the first hour. In experiments carried on for four hours the final phage titre of the supernatant and the final carbohydrate content of the bacterial residue were the same as at the end of the first hour. Tests made at shorter intervals showed that the greatest release of phage took place during the first ten or fifteen minutes of incubation, while less carbohydrate was hydrolysed during this period than during the next one. This may have been due to the fact that the suspensions were not pre-heated before incubation owing to the rapidity of the phage release. Controls showed that the same lag period occurred in the hydrolysis of carbohydrate from heat-killed bacteria which had not previously been coated with phage. Without lysozyme only small increases in phage titre or hydrolysis of bacterial carbohydrate took place. Table V gives the results of four experiments. In Experiment 1 the means of the plaque counts and their standard deviations were calculated, in each case from nine counts of a given dilution. Comparison of the differences between pairs of means was made by the usual statistical methods. The increase in phage titre in the control is not significant, but the titres after 15 and 60 mins. of lysozyme treatment differ significantly from the initial value. These results show that it is possible for phage release to be directly connected with carbohydrate hydrolysis as both occur during the same time interval. But if they are connected one must assume either that incomplete hydrolysis of carbohydrate is sufficient to release phage, since the rate of phage liberation is greater than the rate of carbohydrate disappearance, or that all hydrolysable carbohydrate does not have phage attached to it. Either of these seem reasonable assumptions, but neither is susceptible to proof at present.

DISCUSSION.

The experimental results presented here show that egg-white lysozyme hydrolyses a carbohydrate present in *B. megatherium* M, that it releases phage from its union with heat-killed bacteria during the same time interval, and that after treatment with lysozyme bacteria can no longer adsorb phage. There is no evidence that these events are connected, but there is evidence that the polysaccharides of other bacteria adsorb phages (Bruce White, 1936 ; Burnet and Freeman, 1937 ; Craigie and Brandon, 1936 ; Gough and Burnet, 1934) ; and in the case of one other phage lysis of the bacteria by phage is accompanied by hydrolysis of bacterial carbohydrate (Pirie, 1939). This collected evidence suggests that release of phage during lysis may be due, at

least partly, to break-up of the union between phage and a bacterial polysaccharide. Work is being continued with the aim of separating this polysaccharide from *B. megatherium* M.

SUMMARY.

(1) Phage can be released from its union with heat-killed *B. megatherium* M by treatment with egg-white lysozyme though not by treatment with pangestin or takadiastase.

(2) Heat-killed *B. megatherium* M which has been incubated with lysozyme can no longer adsorb phage.

(3) Reducing sugars are produced when heat-killed bacteria are incubated with egg-white lysozyme.

(4) The release of phage from heat-killed, phage-coated *B. megatherium* M by lysozyme occurs during the same time interval as the hydrolysis of the bacterial polysaccharide.

I wish to thank Prof. E. Wollman for sending me the strains of bacteria and of phage that I have used in these experiments. The work was begun in Dr. K. Meyer's laboratory, College of Physicians and Surgeons, Columbia University, New York, and I wish to thank him for his interest and help.

REFERENCES.

Burnet, F. M., and Freeman, M.—(1937) *Aust. J. exp. Biol. med. Sci.*, **15**, 49.
Clifton, C. E., Mueller, E., and Rogers, W.—(1935) *J. Immunol.*, **29**, 377.
Craigie, J., and Brandon, K. F.—(1936) *J. Path. Bact.*, **43**, 233.
Fujita, A., and Iwatake, D.—(1931) *Biochem. Z.*, **242**, 43.
Giragossintz, G., Davidson, C., and Kirk, P. L.—(1936–7) *Mikrochemie*, **21**, 21.
Gough, G. A. C., and Burnet, F. M.—(1934) *J. Path. Bact.*, **38**, 301.
Gratia, A.—(1936) *Ann. Inst. Pasteur*, **56**, 307.
Meyer, K., Thompson, R., Palmer, J. W., and Khorazo, D.—(1936) *J. biol. Chem.*, **113**, 303.
Idem, Palmer, J. W., Thompson, R., and Khorazo, D.—(1936) *Ibid.*, **113**, 479.
Miles, A. A., and Misra, S. S.—(1938) *J. Hyg., Camb.*, **38**, 732.
Pirie, A.—(1939) *Brit. J. exp. Path.*, **29**, 99.
Pirie, N. W.—(1936) *Ibid.*, **17**, 269.
White, P. B.—(1936) *J. Path. Bact.*, **43**, 591.—(1937) *Ibid.*, **44**, 276.
Wollman, E., and Wollman, E.—(1936) *Ann. Inst. Pasteur*, **56**, 137.

THE FAILURE OF INTRAPERITONEAL INJECTION OF 3:4-BENZPYRENE SOLUTION TO INCREASE THE GENERAL SUSCEPTIBILITY TO CANCER.

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SEVERAL authors have claimed that the administration of tar increases the general disposition of animals to develop cancer. In many experiments an area was painted with tar, and a different area, either irritated or not, was examined for a remote effect of tar. Such a remote action has been recorded by Murray (1923), Lipschütz (1924), Kreyberg (1927), Akimoto (1929), Polettini (1929), Schabad (1930), and others. Negative findings have been reported by Cramer (1926), and Bonciu and Guérin (1931). In other experiments tar was injected and a remote area of the skin or remote site of the subcutaneous tissue was irritated. Malignant growth at the irritated foci is described by Maisin and Masse (1925), Beck (1927), and Klee-Rawidowicz (1932). No such remote effect has been shown in the case of 3 : 4-benzpyrene, but the inhibiting effect of carcinogenic hydrocarbons on the growth of transplanted, induced, and spontaneous tumours has been described by Haddow (1935, 1938*a*, 1938*b*) and others.

The purpose of this investigation was to test the possible effect of intraperitoneal injections of 3 : 4-benzpyrene on the cancer susceptibility of mice.

EXPERIMENTS.

110 stock mice were injected intraperitoneally with 1 mg. 3 : 4-benzpyrene in 0·5 ml. olive oil. Ten of these mice were kept as controls. The remainder were treated in the following groups :

(*a*) The skin of 10 mice was cauterized under anaesthesia with the red-hot head of a pin, and the subcutaneous tissue of another 10 mice was exposed by incision under anaesthesia and cauterized in the same way, three months after the 3 : 4-benzpyrene injection. Ten cauterized mice served as controls.

(*b*) Twenty mice had the right hind leg irradiated with X-rays (150 kv., 6 ma., 23 cm. distance, open field, 0·3 mm. copper with 1·0 mm. aluminium, 14 minutes 45 seconds, 600 r.) three months after the intraperitoneal injection. Ten mice were irradiated in the same way, as controls.

* Working under a full-time grant from the British Empire Cancer Campaign.

(*c*) Forty mice were irradiated once a week with an erythema dose of ultra-violet rays on the epilated interscapular region, three months after the 3 : 4-benzpyrene injection. Seven mice were irradiated only, as controls.

(*d*) Twenty mice were painted with a 0·3 per cent. solution of 3 : 4-benzpyrene in benzene twice a week, starting thirty days after the intraperitoneal injection of 3 : 4-benzpyrene. Twenty mice were painted in the same way, as controls.

RESULTS.

Twenty-six mice developed intraperitoneal sarcomata between the fourth and the eighth months, and were killed. Eighty-six mice survived for six months, and 39 for twelve months, after the injection of 3 : 4-benzpyrene. Two polypi of the anal canal occurred in the mice injected with 3 : 4-benzpyrene and treated with X-rays. One polyp regressed, and the other showed histologically the structure of a simple polyp, with hyperplasia of the mucous membrane. This result is considered to have no relation to the susceptibility to cancer. No tumours appeared in any of the cauterized or irradiated mice, and the development of tumours in the painted mice was not accelerated.

DISCUSSION.

In these experiments the dose of 3 : 4-benzpyrene used was intentionally small, in order to avoid possible inhibiting effects on any tumour that might arise. On the other hand, it is unlikely that the tar, used in other experiments of similar nature, contained as much 3 : 4-benzpyrene as was used in this investigation. Cook required 2 tons of tar for the isolation of 50 g. 3 : 4-benzpyrene. The general inhibiting effect of 3 : 4-benzpyrene affecting body growth as well as tumour growth seems to be of toxic origin, according to Haddow's own explanation.

There is thus a discrepancy between the results of the administration of tar and of 3 : 4-benzpyrene. There is evidence that tar increases the general susceptibility to cancer. There is no such evidence in the case of 3 : 4-benzpyrene. But there are other qualitative differences between the action of tar and of 3 : 4-benzpyrene. The skin of the rabbit responds readily to the carcinogenic action of tar, but is very refractory to the same action of 3 : 4-benzpyrene. According to a personal communication by Dr. Peacock, 3 : 4-benzpyrene injected in fowls in quantities up to 10 mg. has so far failed to induce sarcoma during periods up to three years from the time of injection, while tar induced tumours in over 30 per cent. (Peacock, 1933, 1935). The different actions of tar and 3 : 4-benzpyrene on the skin of rabbits led Berenblum (1939) to the conclusion that tar may contain other carcinogenic substances besides 3 : 4-benzpyrene. The same conclusion would explain the differences in the general susceptibility to cancer of mice and in the local susceptibility of fowls, treated with tar and with 3 : 4-benzpyrene.

On the other hand, pure 3 : 4-benzpyrene, dissolved in olive oil, may act quite differently from the 3 : 4-benzpyrene occurring in tar. As far as local administration is concerned, the hydrocarbon may be longer retained by the complex mixture of tar than by a simple solution. As far as the general effect is concerned, it has been shown that tar and 3 : 4-benzpyrene act differently.

3 : 4-benzpyrene, parenterally injected, becomes dissolved in serum and fats, metabolized by the liver, and excreted in the bile (Peacock, 1936). What becomes of the 3 : 4-benzpyrene fraction of the tar is unknown. Burrows (1932) gives evidence that electro-negative colloids become concentrated in areas of inflammation. It seems possible that some parts of the injected tar may enter the blood stream in a colloidal state and thus would tend to be concentrated in areas inflamed by irritation. If 3 : 4-benzpyrene is associated with such colloidal particles, it might induce tumours in the irritated regions.

SUMMARY.

Mice were injected intraperitoneally with 1 mg. 3 : 4-benzpyrene, dissolved in 0·5 ml. olive oil, and three months later the skin or the subcutaneous tissues were irritated by (*a*) cauterization : (*b*) X-rays ; (*c*) ultra-violet rays ; no tumours occurred at the irritated sites.

Mice injected similarly with 3 : 4-benzpyrene and then painted on the nape of the neck with 3 : 4-benzpyrene solution developed tumours in the painted areas, but the local carcinogenic effect was not accelerated.

REFERENCES.

Akimoto, K.—(1929) *Z. Krebsforsch.*, **30**, 428.
Beck, S.—(1927) *Ibid.*, **24**, 278.
Berenblum, I.—(1939) *Ann. Rep. Brit. Emp. Cancer Campgn.*, **16**, 213.
Bonciu, C., and Guérin, M.—(1931) *Bull. Ass. franç. Cancer*, **20**, 391.
Burrows, H.—(1932) ' Some Factors in the Localization of Disease in the Body,' London (Baillière, Tindall & Cox).
Cramer, W.—(1926) *Brit. J. exp. Path.*, **7**, 1.
Haddow, A.—(1935) *Nature*, **136**, 868.—(1938*a*) *J. Path. Bact.*, **47**, 567.—(1938*b*) *Ibid.*, **47**, 581.
Klee-Rawidowicz, E.—(1932) *Z. Krebsforsch.*, **38**, 35.
Kreyberg, L.—(1927) *Brit. J. exp. Path.*, **8**, 352.
Lipschütz, B.—(1924) *Wien. klin. Wschr.*, **37**, 1258.
Maisin, J., and Masse, G.—(1925) *C.R. Soc. Biol., Paris*, **93**, 449.
Murray, J. A.—(1923) *Sci. Rep. Imp. Cancer Res. Fd., Lond.*, **8**, 75.
Peacock, P. R.—(1933) *J. Path. Bact.*, **36**, 141.—(1935) *Amer. J. Cancer*, **25**, 37.—(1936) *Brit. J. exp. Path.*, **17**, 164.
Polettini, B.—(1929) *Tumori*, **15**, 306.
Schabad, L. M.—(1930) *Z. Krebsforsch.*, **31**, 621.

PYRIDINE-3-SULPHONIC ACID AND ITS AMIDE AS INHIBITORS OF BACTERIAL GROWTH.

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THE generalization that many compounds inhibitory to growth of living organisms act by interfering specifically with essential metabolites was expressed by Fildes (1940), and a particular type exemplified by Woods (1940) in the action of sulphanilamide. Evidence was given by Woods that this compound acts by preventing the utilization of p-aminobenzoic acid, considered to be an essential metabolite, by competing with it (by reason of its similarity in chemical structure) for the enzymes involved in its further utilization.

This paper presents work based on the hypothesis of Fildes. Nicotinic acid is a metabolite known to be essential to staphylococcus (Knight, 1937) and to proteus (Fildes, 1938), and therefore pyridine sulphonic acid amide, which is related to nicotinic acid as sulphanilamide is to p-aminobenzoic acid, was tested as an inhibiting agent. The investigations were extended to inhibition of growth promoted also by nicotinamide and cozymase and inhibited also by pyridine sulphonic acid and other nicotinic acid analogues. The effects of the inhibitors were examined also in relation to *Bact. coli*, which does not require added nicotinic acid for growth.

EXPERIMENTAL.

Materials.

Materials were those used by Knight and McIlwain (1938) with the following exceptions:

Pyridine-3-sulphonic acid and *amide* were prepared according to Machek (1938) and treated with H_2S as a precaution against extraneous inhibitory effects from mercury used as catalyst.

Cozymase.—A specimen prepared by Dr. B. C. J. G. Knight and Dr. G. D. Greville was identical in growth effect with one prepared by Dr. J. L. Still and was used throughout. It is not necessarily considered a pure compound, but as a nicotinamide derivative of " V " factor activity (Fildes, 1938).

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Media and conditions of growth.

Bact. coli and *Proteus vulgaris* were grown in the modified ammonium lactate medium of Fildes (1940) from inocula of about 1000 organisms from a previous test. Each test solution contained the experimental quantities of growth promoter and inhibitor in 10 ml. of medium in 50 ml. conical flasks incubated at 37° in air containing 5 per cent. CO_2.

The deficient staphylococcus medium consisted of the amino acid basal medium of Gladstone (1939) containing aspartic acid, valine, leucine, alanine, glutamic acid, *iso*leucine, phenylalanine, lysine, glycine, proline, hydroxyproline, tyrosine, arginine, histidine and buffer with the addition of $(NH_4)_2SO_4$, NH_4Cl, $MgSO_4$, $Fe(NH_4)_2(SO_4)_2.6H_2O$, cystine, methionine, tryptophane, aneurin, riboflavin and glucose in the quantities described by McIlwain (1940) as a solution sterilized by filtration. Each tube contained in 10 ml. the experimental amounts of growth promoter and inhibitor and an inoculum of about 10,000 organisms from a 24-hour broth-agar slope, and was incubated in an upright position in air containing 5 per cent. CO_2.

Effects of inhibiting agents.

In finding the range of action of a growth inhibitor it was initially tested over a wide range of concentrations in 10-fold dilutions. In order to investigate the type of inhibition, the important range was studied in greater detail employing the 5-fold dilutions seen in the majority of the tables. Growth was estimated by the opacity of the solutions. A reference titration of growth promoting agent (with staphylococcus and proteus) or a reference flask (with coli) was included with each test, and the effect of an inhibitor was in reality estimated by the null method of comparing growth in its presence with the reference titration of the same test. The ++ signs of the tables are proportional to these opacities ; ? indicates just visible growth.

In most cases the highest concentration of inhibitor investigated was 10^{-2} *M* ; in the instances in which higher concentrations were used and inhibition occurred it was found to be non-specific in the sense that it could not be reversed by adding nicotinic acid or a derivative, and was therefore considered outside the scope of the present work.

NICOTINIC ACID ANALOGUES AS AGENTS INHIBITING GROWTH OF STAPHYLOCOCCUS.

Knight and McIlwain (1938) in studying the deviations from the structure of nicotinamide compatible with its functioning as growth factor for staphylococcus, incidentally recorded that a number of compounds which did not support growth in nicotinamide deficient media were not inhibitory in concentrations of 10^{-4} *M* to growth in the presence of 10^{-6}–10^{-7} *M* nicotinamide. These experiments were performed in order to ensure that absence of growth was not due to extraneous inhibitory action. An investigation of inhibitory action *per se* necessitated experiments over a wider range of concentrations, which were carried out in the cases of suitably blocked or modified nicotinic acid analogues.

TABLE I.—*Effect of Nicotinic Acid Analogues on Staphylococcus Growth.*

Nicotinamide $\times 10^{-6}M$.	Compound investigated as inhibitor, $10^{-2}M$.	Growth after (hours) 20.	28.	45.
0	0	0	0	0
2	0	++	+++	++++
0·4	0	+	++	++
0·08	0	?	+	++
0·016	0	0	?	+
0	2.4.6-trimethyl-pyridine-3.5-dicarboxylic acid	0	0	0
2	Ditto	++	+++	++++
0·4	,,	+	++	++
0·08	,,	?	+	++
0	Trigonelline *or* coramine *or* quinolinic acid	?	+ to ++	++++
2	Ditto	++	++ to +++	++++
0·4	,,	+	++ to +++	++++
0·08	,,	?	+ to ++	++++
0	Nicotine	?	++	++++
2 to 0·08	,,	?	++	++++

Table I shows that growth was indifferent to the presence of 2.4.6-trimethylpyridine-3.5-dicarboxylic acid, a fully substituted nicotinic acid. Quinolinic acid, a nuclear substituted nicotinic acid; trigonelline, an N-substituted nicotinic acid, and coramine, the diethylamide, were at first without action and later served as sources of nicotinic acid. At lower concentrations not included in the table, these compounds neither accelerated nor retarded growth. Nicotine at 2.10^{-3} *M* behaved similarly, but at 10^{-2} *M* an initial inhibition was observed covering the first 28 hours of growth; the compound later stimulated growth. Pyridine-3-sulphonic acid and its amide inhibited throughout and are described in detail below.

Pyridine-3-sulphonamide Inhibition of Nicotinamide and Nicotinic Acid Promoted Growth.

Table II shows a titration of nicotinamide reaching from the lowest concentration at which maximal growth is attained (c. 10^{-6} *M*) to the highest concentration at which no growth occurs (c. 3×10^{-9} *M*). Maximal growth was reached between 26 and 43 hours, after which the mass of growth remained constant for two days before gradual diminution from autolysis. Growth within this range was roughly proportional to the quantity of nicotinamide present; with higher or lower concentrations this was not the case.

TABLE II.—*Pyridine Sulphonamide Inhibition of Nicotinamide-Promoted Staphylococcus Growth.*

Nicotinamide $\times 10^{-6} M$.	Pyridine sulphonamide $\times 10^{-2} M$.	Growth after (hours) 19.	26.	43 to 4 days.
0	0	0	0	0
1	0	+	+++	++++
0·2	0	+	+++	++++
0·04	0	+	++	+++
0·008	0	?	+	+++
0·0016	0	0	?	+
0·00032	0	0	0	0 or ?
1	1	+	++	+++
0·2	1	?	+	+++
0·04	1	0	?	++
0·008	1	0	0	0
0·2	0·2	+	++	+++
0·04	0·2	?	+	+++
0·008	0·2	0	?	++
0·0016	0·2	0	0	0
0·04	0·04	+	++	+++
0·008	0·04	?	+	++
0·0016	0·04	0	0	+
0·00032	0·04	0	0	0

Addition of pyridine-3-sulphonamide results in a reduction of growth in a characteristic manner (referred to later as Type I inhibition), of which the following points may be noted: (1) Addition of 10^{-2} *M* sulphonamide to media containing 10^{-6} *M* nicotinamide results in reduction of growth to the level given in absence of the sulphonamide by 4×10^{-8} *M* nicotinamide. This reduction of the effective concentration of nicotinamide to 1/25 of the amount actually present is caused also by 10^{-2} *M* pyridine sulphonamide in the presence of the other concentrations of nicotinamide investigated, and (2) holds throughout the period of observation, which was extended until considerable lysis had occurred; it must be considered a characteristic feature of 10^{-2} *M* pyridine sulphonamide solutions. (3) The effect of lowering the concentration of pyridine sulphonamide is to reduce gradually its inhibitory action; expressed in the above manner, the effect of 2×10^{-3} *M* pyridine sulphonamide is to reduce the effective concentration of nicotinamide to 1/5 of that actually present, while 4×10^{-4} *M* has very little action. (4) In no case is growth promoted by nicotinamide retarded by pyridine sulphonamide; the reduced maxima are reached in the same time of one to two days. The results of Table II can also be expressed by saying that within the range of concentrations recorded, a given mass of growth is produced, not by a given concentration of nicotinamide, but by a nicotinamide concentration which is a given fraction of the concentration of pyridine sulphonamide present. Thus when this fraction is 10^{-4} growth in each case is represented by +, ++

and +++ after 19, 26, and 43 hours respectively. These characters are summarized in Table V, which contrasts Type I with other inhibitions described later.

The action of pyridine sulphonamide on growth of staphylococcus promoted by *nicotinic acid* is very much less than its action on growth promoted by the amide, though it was of the same type and exhibited the characters 1 to 4 in so far as they could be observed. With growth promoted by *cozymase* a different type of inhibition (Type II) was produced, which is discussed in detail below in relation to inhibitions which exhibit it more completely. The inhibitions are compared quantitatively in Table VI. No inhibition was found in broth or peptone media, known to contain excess of nicotinamide-like growth factors.

Pyridine Sulphonic Acid Inhibition.

The action of this inhibitor on growth promoted by *nicotinamide*, shown in Table III, is in sharp contrast to the results of Table II. (1) In the first place, no result can be expressed simply by saying that the effect of the inhibitor is to reduce the effective concentration of nicotinamide. (2) The degree of inhibition changes with time, pyridine sulphonic acid becoming less effective as an inhibitor after some days' growth. (3) Growth is retarded as well as reduced, and retarded more by the higher concentrations of pyridine sulphonic acid than by the lower. (4) After the period of retardation, growth in the presence of a given quantity of sulphonic acid takes place more quickly in tubes containing higher concentrations of nicotinamide than in those containing lower concentrations. This is termed Type II inhibition (Table V).

TABLE III.—*Pyridine Sulphonic Acid Inhibition of Nicotinamide-Promoted Staphylococcus Growth.*

Nicotinamide $\times 10^{-6}M$.	Pyridine sulphonic acid $\times 10^{-2}M$.	Growth after (hours and days)				
		23.	30.	47.	3.	5 and 6.
0	0	0	0	0	0	0
1	0	+++	++++	+++	+++	+++
0·2	0	+++	+++	++++	+++	+++
0·04	0	++	+++	+++	+++	+++
0·008	0	+	++	++	++	+
0·0016	0	0	?	?	?	?
0·00032	0	0	0	0	0	0
1	1	0	0	0	?	+++
0·2	1	0	0	0	0	+++
0·04	1	0	0	0	0	0
0·2	0·2	0	+	+++	+++	+++
0·04	0·2	0	0	++	++	+++
0·008	0·2	0	0	0	0	?
0·0016	0·2	0	0	0	0	0
0·04	0·04	?	++	+++	+++	+++
0·008	0·04	0	0	?	?	?
0·0016	0·04	0	0	0	0	?

Nicotinic acid as growth-promoting agent for staphylococcus is required in higher concentrations than its amide ; using a maximum concentration of nicotinic acid of 5.10^{-5} *M*, similar results were obtained in that growth was retarded and reduced, but not merely reduced as though the concentration of nicotinic acid available was a definite fraction of that added ; inhibition was partly overcome after some days, more quickly when more nicotinic acid was present. Growth promoted by *cozymase* was also inhibited by pyridine sulphonic acid in the same manner ; the extent of these inhibitions is recorded in Table VI. Pyridine sulphonic acid considerably retarded growth of staphylococcus in a peptone medium, but had only little effect in broth media.

Summarizing these results with staphylococcus two types of inhibition have been encountered, and it would appear that the type of inhibition which operates in a given case depends upon both the growth-promoting and growth-inhibiting agents and not upon either singly. Alteration of the concentration of either agent alters the degree of inhibition and may remove it altogether, but does not alter its type while it exists.

INHIBITION OF GROWTH OF *PROTEUS*.

Pyridine-3-sulphonamide.

Inhibition of growth promoted by nicotinic acid was, as with staphylococcus, negligible. The very small inhibition was evident only during the first day. Inhibition of *nicotinamide*-promoted growth was smaller than was the case with staphylococcus, and unlike it, changed with time, usually having disappeared on the third or fourth day, earliest with higher concentrations of nicotinamide. In this the inhibitions are similar to the Type II recorded above for staphylococcus with pyridine sulphonic acid, except that in no case is the inhibition so complete and lasting. Inhibition of *cozymase*-promoted growth was also small and covered only the first day.

Pyridine-3-sulphonic acid.

The effect of pyridine sulphonic acid on *nicotinic acid*-promoted proteus growth (Table IV) is to produce a type of inhibition very different from Type I but in some ways similar to Type II. The following points may be observed in comparison with those noted for Types I and II : (1) As in Type II, no result can be expressed as a fractional reduction in the concentration of growth factor, and (2) the degree of inhibition changes with time. (3) Growth is retarded, but there is not the gradual reduction of action found in Type I on lowering the inhibitor concentration. (4) The most marked characteristic is that after the period of retardation growth in the presence of a given quantity of sulphonic acid takes place independently of the concentration of nicotinic acid. This is referred to as Type III inhibition (Table V), and is exhibited again in the pyridine sulphonic acid inhibition of *cozymase*-promoted proteus growth. This was even more powerfully inhibited, retardation comparable to that given by 10^{-2} *M* sulphonic acid when nicotinic acid was used as promoter being given by 2×10^{-3} *M* when cozymase was employed.

TABLE IV.—*Pyridine Sulphonic Acid Inhibition of Nicotinic Acid- and of Nicotinamide-Promoted Growth of* Proteus.

Promoter $\times 10^{-5}$ *M.*	Pyridine sulphonic acid $\times 10^{-2}$ *M.*	Growth after (hours and days) 22.	29–30.	47.	3–4.
0	0	0	0	0	0
Acid :					
1	0	—	+	+++	+++
0·2	0 *or* 0·2	—	+	+++	+++
0·4	0 *or* 0·2 *or* 0·04	—	+	+++	+++
0·008	0 *or* 0·2 *or* 0·04	—	+	++	++
0·0016	0 *or* 0·2 *or* 0·04	—	+	+	+
0·00032	0 *or* 0·04	—	0	0	0
1	1	—	0	0	++ to +++
0·2	1	—	0	0	+++
0·04	1	—	0	0	+++
0·008	1	—	0	0	++
Amide :					
1	0	0	+	+++	++++
0·1	0	0	+	+++	++++
0·01	0	0	+	++	++
0·001	0	0	0	0	0
1	1	?	++	+++	++++
0·1	1	?	++	+++	++++
1	0·1	?	++	+++	++++
0·1	0·1	0	+	+++	+++
0·01	0·1	0	+	++	++
1	0·01	0	++	+++	++++
0·1	0·01	0	+	+++	++++
0·01	0·01	0	++	++	++
0	1, 0·1 *or* 0·01	0	0	0	0

Pyridine sulphonic acid added to *nicotinamide*-containing media for proteus growth showed the unusual effect of accelerating growth. Acceleration was evident only during the first day (Table IV) and did not lead to increased mass of growth ; it occurred most definitely with the highest concentration (10^{-2} *M*). The acid itself was not found capable of causing growth, but has been found to

accelerate also the growth of coli and streptococcus under certain conditions. This phenomenon is undoubtedly of importance, and such borderline cases indicate that there is only a narrow line of demarcation between the growth-inhibiting and growth-promoting action of a growth-factor analogue. In the extreme cases it may result in an " inhibitor " acting as a growth-promoting, and not merely accelerating, agent (*cf.* Lwoff and Querido, 1939).

It was thought possible that the late appearance of growth characteristic of Type III inhibition might be due to mutation of the organism to a pyridine sulphonic acid-resistant strain, but growth on subculture from the proteus delayed by 10^{-2} *M* pyridine sulphonic acid in the presence of 10^{-6} *M* nicotinic acid was equally inhibited nor would it grow in the absence of nicotinic acid.

INHIBITION OF GROWTH OF *BACT. COLI.*

As this organism does not require added nicotinamide-like compounds for growth the technique was changed, inasmuch as it was first attempted to prevent or delay growth, and then to reverse the inhibition with nicotinic acid *et al.* Inhibition with pyridine sulphonamide was small in 10^{-2}*M* solutions and non-existent at 1/5 or 1/25 of this concentration. Higher concentrations gave definite inhibition which was non-specific in the sense referred to on p. 137. The small inhibition caused by 10^{-2} *M* sulphonamide was completely reserved by nicotinic acid or its amide. 10^{-2} *M* pyridine sulphonic acid completely inhibited growth of coli ; 5×10^{-3} *M* usually did so, but neither of these inhibitions was affected by nicotinic acid, amide, or cozymase. Concentrations of 2×10^{-3} *M* and less pyridine sulphonic acid were without action on growth.

DISCUSSION.

Type of Inhibition.

If one generalizes from Fildes' evidence (1938) that nicotinic acid used by *Proteus* during growth is built up to compounds of at least the complexity of pyridine nucleotides, one has a picture not merely of a given compound, such as nicotinic acid, being a critical factor in nicotinic acid limited growth, but rather of a critical series of reactions building nicotinic acid to at least coenzyme level. These reactions are not normally susceptible to direct investigation in growth experiments, their occurrence being deduced from the disappearance of a simple compound and the appearance of a more complex, as in the case of Fildes' experiments, or from a greater efficacy of the same molar concentration of the more complex compound (e.g. Knight and McIlwain, 1938, on nicotinic acid and its amide in staphylococcus growth). It is, however, with such a series of reactions that pyridine sulphonic acid and its amide appear to interfere. The results given above can be interpreted to a considerable degree in terms of enzyme inhibition. Complicating influences are that (*a*) the whole of the reactions of the living cell are involved to a greater or lesser degree, and (*b*) the organism may adapt itself to its unusual circumstances.

TABLE V.—*Characters of the Different Types of Inhibition Observed in Bacterial Growth.*

	Type I.	Type II.	Type III.
Inhibition manifested as	Reduction in growth No retardation	Sometimes reduction Retardation	No reduction. Retardation.
Time	Growth remains in same reduced ratio	Inhibition decreases or disappears	Inhibition decreases or disappears.
Reduction of concentration of growth promoter	Inhibition more complete (but growth reduced to the same fraction)	Inhibition more complete	Inhibition unchanged.
Concentration of promoter and inhibitor changed in same ratio	Inhibition unchanged	Inhibition usually changes	Inhibition increases with increasing values.

Type I inhibition can then be recognized as comparable to competitive inhibition, in that inhibition is greater the higher the concentration of inhibiting agent and the lower the concentration of substrate. In the case of staphylococcus, growth over the range of concentrations of Table II is increased by increasing the concentration of promoting agent in the same manner as it is decreased by increasing the inhibitor concentration. If direct proportionality, shown by Querido *et al.* (1939) to exist between nicotinic acid concentration and growth of *proteus*, is assumed, growth $\propto \frac{[\text{promoter}]}{[\text{inhibitor}]}$. This is not necessarily the case with competitive enzyme inhibition.

Type III inhibition is, on the other hand, comparable to non-competitive inhibition in that inhibition is dependent only on concentration of the inhibitor and independent of that of the growth-promoting agent.

The explanation of the different types of inhibition of bacterial growth may be those suggested for enzyme kinetics—i.e. that in competitive inhibition growth promoter and inhibitor are competing for the same centre on the enzyme surface, while in non-competitive inhibition the inhibitor is adsorbed elsewhere on the enzyme. It is not supposed that the substrate inhibited is necessarily the added growth factor itself.

Type II inhibition.—Of the other instances of inhibition investigated above, several do not fit clearly into either Type I or Type III, which must be regarded as extremes of a series of possible types of inhibition. The pyridine sulphonic acid-inhibition of nicotinic acid- or nicotinamide-promoted growth of staphylococcus and the pyridine sulphonamide inhibition of growth of *Proteus* promoted by nicotinamide are of this intermediate Type II. Unlike Type III, inhibition is less in the presence of a higher concentration of the growth-promoting agent. Unlike Type I, growth is not reduced merely as though a given fraction of the growth-promoting agent were effective and the degree of inhibition changes with time. Clearly in so complicated a process as the utilization of nicotinic acid at least partly for the synthesis of pyridine nucleotides many reactions must be involved, and unless the action of the inhibitor is confined to one of these, or inhibits several in the same manner, intermediate examples such as these are to be expected. However, the inverse

proportionality between growth-promoter and inhibitor concentrations observed in Type I is not a necessary feature of competitive inhibition, but only of those in which increase in promoter concentration affects growth in the same manner as decrease in inhibitor concentration. Type II may therefore represent a different form of competitive inhibition, or the superposition of competitive and non-competitive inhibitions.

Reactions Inhibited.

The inhibitors studied are believed to affect the utilization of nicotinic acid or its derivatives, as these compounds will remove or reduce the inhibition. The medium used for growth of staphylococcus already incorporates many other growth factors; negative results of attempted reversal by the other known nutritionally important pyridine derivative, adermin, were obtained in all the instances in which it was tried. The clearest evidence for the specificity of the inhibition is, however, given in Table IV, in which it is seen that pyridine sulphonic acid inhibits growth of *Proteus* promoted by nicotinic acid considerably, but hardly affects its growth promoted by nicotinamide; staphylococcus growth presents a similar instance. Such different degrees of inhibition with different sources of pyridine compounds enable suggestions to be made concerning their utilization by the organism. If nicotinic acid were used solely for preparation of nicotinamide and then cozymase, inhibition of a particular organism by a given inhibitor should decrease or disappear when either of the latter two compounds are used in place of nicotinic acid as source of growth-promoting factors, as there would be less opportunity for interference in reactions.

Table VI shows that pyridine sulphonic acid inhibits growth of *Proteus* considerably when nicotinic acid is used as growth-promoting agent, but not at all when corresponding concentrations of nicotinamide are used. This suggests the action of pyridine sulphonic acid to be localized at the reaction converting nicotinic acid to its amide, or at a similar stage of amide formation from a higher derivative. Growth promoted by cozymase is, however, much more strongly inhibited even than that promoted by corresponding concentrations of nicotinic acid. This would indicate that nicotinamide is not used by *Proteus* solely for the synthesis of cozymase or cozymase derivatives. As the formation of the necessary compounds from nicotinamide can proceed unimpeded by pyridine sulphonic acid, the inhibition must at least in part occur during degradation of cozymase to smaller units used in synthesis. Pyridine sulphonamide inhibition of *Proteus* is, as was the case with the acid, greatest with the simple compound to which it bears the closest resemblance, in this instance with nicotinamide, though it is not possible to relate it to any particular reaction.

With staphylococcus also, pyridine sulphonamide inhibits nicotinamide-promoted growth more powerfully than that promoted by the acid. Inhibition of growth promoted by cozymase is sharply differentiated as it is not only more intense, but involves a different type of inhibition, Type II, whereas those of the acid and amide are of Type I. This is taken to indicate that different reactions are involved in the inhibition and that, with staphylococcus

also, added nicotinic acid is not used solely for the synthesis of cozymase. Inhibition by pyridine sulphonic acid is, on the other hand, always of Type II, and affects equimolecular concentrations of all three growth promoters to the same degree. The inhibition is more intense than that of the sulphonamide, and may affect most powerfully a common reaction at a stage higher than cozymase.

TABLE VI.—*Summary of Type and Extent of Inhibition.*

ret. = Retardation of growth, expressed in days.

red. = Reduction of growth, expressed as the fraction of growth promoter actually present to which the inhibited growth corresponds. Absence of reduction is thus given by the figure 1.

Concentrations are expressed in molarities. The highest concentration of promoter in each set is approximately the optimal concentration for normal growth.

Type of inhibition is explained on pp. 139, 140. When the inhibition is not sufficiently large to be well characterized, the probable type is given in parentheses.

Promoter. (Nicotinic acid, amide or cozymase.)			Inhibitor. (Pyridine sulphonic acid or its amide.)						Type.
			10^{-5}.		2×10^{-5}.		4×10^{-4}.		
			ret.	*red.*	*ret.*	*red.*	*ret.*	*red.*	
Staphylococcus :									
Acid,	10^{-5}–8×10^{-8}	.	Amide	0	1/5–1	0	1	0	1	(I)
Amide,	10^{-6}–10^{-9}	.	,,	0	1/25	0	1/5	0	1/5–1	I
Cozymase,	10^{-7}	.	,,	1	1/5–1/25	0	1/5	..	..	II
	2×10^{-8}	.	,,	4–5	1/5–1	1	1/5	..	..	
	4×10^{-9}	.	,,	>5	..	4–5	0	..	..	
Acid,	10^{-5}	.	Acid	1–2	1	<1	1	..	..	II
	2×10^{-6}	.	,,	3	1/5	1	1	1	1	
	4×10^{-7}	.	,,	5	1/5	2	1	1	1	
Amide,	2×10^{-7}	.	,,	4	1/5	1	1/5	..	..	II
	4×10^{-8}	.	,,	>6	..	3–4	1/5–1	<1	1/5–1	
	8×10^{-9}	.	,,	>6	..	4–5	1/5	1–2	1/5	
Cozymase,	10^{-6}	.	,,		..	1	1	<1	1	II
	2×10^{-7}	.	,,		..	2	1/5–1	3–4	1	
	4×10^{-8}	.	,,		..	4	1/5–1	4	1	
Proteus :									
Acid,	5×10^{-7}–2×10^{-8}		Amide	<1	1	0	1	..	..	(III)
Amide,	2×10^{-7}	.	,,	2	1	0	1	..	..	II
	4×10^{-8}	.	,,	3	1	0	1	0	1	
	8×10^{-9}	.	,,	>4	..	3	1/5–1	2	1	
Cozymase,	5×10^{-7}	.	,,	>1	1	0	1	..	..	(II)
	10^{-7}	.	,,	1	1/5–1	0	1	..	..	
Acid,	10^{-5}–8×10^{-8}		Acid	2	1	0	1	0	1	III
Amide,	10^{-5}–10^{-7}	.	,,	0	1	0	1	0	1	None
Cozymase,	10^{-5}–8×10^{-8}		,,	>6	..	2	1	1	1	III
Coli :									
0		.	Amide	1	1	<1	1	..	..	
Amide,	10^{-6}	.	,,	0	1	0	1	..	..	
0		.	Acid	>6	..	0	1	0	1	
Acid, amide *or* cozymase	10^{-5}–2×10^{-8}		,,	>6	..	0	1	0	1	

SUMMARY.

The rational approach to chemotherapy suggested in this department has led to compounds related to nicotinic acid being investigated as bacterial inhibitors. Three types of inhibition have been recognized and are characterized briefly in Table V. Results with staphylococcus, *Proteus* and *Coli* in media of known composition, summarized in Table VI, are consistent with the theory advanced, and have enabled suggestions to be made relating to the nicotinic acid metabolism of two of these organisms.

I have pleasure in acknowledging suggestions and advice received from Dr. P. Fildes and from other members of this department.

REFERENCES.

FILDES, P.—(1938) *Brit. J. exp. Path.*, **19**, 239.—(1940) *Ibid.*, **21**, 67.
GLADSTONE, G. P.—(1939) *Ibid.*, **20**, 189.
KNIGHT, B. C. J. G.—(1937) *Biochem. J.*, **31**, 731.
Idem AND MCILWAIN, H.—(1938) *Ibid.*, **32**, 1241.
LWOFF, A., AND QUERIDO, A.—(1939) *C.R. Soc. Biol., Paris*, **130**, 1569.
MACHEK, G.—(1938) *Mh. Chem.*, **72**, 77.
MCILWAIN, H.—(1940) *Brit. J. exp. Path.*, **21**, 25.
QUERIDO, A., LWOFF, A., AND LATASTE, C.—(1939) *C.R. Soc. Biol., Paris*, **130**, 1580.
WOODS, D. D.—(1940) *Brit. J. exp. Path.*, **21**, 74.

INFLUENZA VIRUS INFECTIONS OF THE CHICK EMBRYO LUNG.

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IN a paper on meningococcal infections of the chick embryo, Buddingh and Polk (1939) describe a method of inoculation into the amniotic cavity by which infection of the lungs and meninges may be produced. It seemed possible that this technique might find some interesting applications in regard to influenza virus. We first used two strains partially adapted to chorioallantoic growth, and found that infection of the lungs occurred, and that these organs contained much virus. Chorioallantoic infection by these strains gave no virus in the lungs or other internal organs of the embryo. Most of the influenza strains we possess were then examined for their power to infect by the amniotic route. All produced specific lung infections, with the possible exception of the highly virulent egg-adapted strains of "Melbourne" and "W.S.," which produce rapidly fatal generalized infections of the embryo.

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The most important finding was that wholly unadapted early ferret passage virus could produce typical infections of the embryo lung. We had available glycerolated material from a ferret inoculated with human throat washings containing influenza virus in June, 1939. This first passage material produced typical embryo lung lesions, and it seems a reasonable deduction that virus direct from human beings would do the same. We have not yet had an opportunity to test this in practice.

The new method has already allowed some interesting findings in regard to influenza viruses, but its greatest potential importance is as a possible means for the isolation of other respiratory viruses. In view of the opinion recently expressed by Stuart-Harris *et al.* (1940) that there are several other

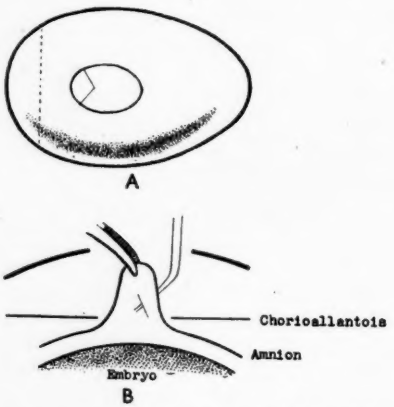


Fig. 1.—Diagrams to indicate (A) the method of drilling the egg-shell and (B) of making the amniotic inoculation.

types of virus infection still unsorted from the clinical group of " influenzas," it seems advisable to publish a preliminary account of the method at once, so as to make it available to other workers.

TECHNIQUE.

Fertile white leghorn eggs are used after preliminary incubation for twelve days. The position of the embryo is determined by transillumination and marked on the shell. This is not always easy, owing to the heavy shadows cast by the yolk sac, but with experience 80–90 per cent. of correct localizations can be obtained. An oval opening, 2 cm. × 1·5 cm., is drilled in the shell and two further cuts made to give a roughly diamond-shaped area at one end of the oval. The inoculation follows the general lines of the chorioallantoic technique (Burnet, 1936). After a small hole has been drilled into the air sac, the diamond-shaped piece of shell is removed. A slit is made in the shell membrane and a drop of sterile saline placed on the slit. Suction is now applied by a teat on the hole into the air sac and the chorio-allantois drawn down. The drop of saline makes this procedure much more gentle than the

original method, and avoids hæmorrhage from the chorio-allantoic capillaries. The rest of the oval area of shell is now removed and the shell membrane clipped away with scissors. The position of the embryo is located and the chorio-allantois over it is picked up with fine forceps. An opening is now made with scissors, care being taken to avoid damage to the underlying amnion. The inoculation is made by means of a Pasteur pipette drawn out to a sharp point. About 0·05 c.c. of inoculum is taken up, and then about 0·1 c.c. of air. The amnion is picked up with forceps through the opening in the chorio-allantois and drawn gently taut. The tip of the capillary is pushed through the base of the " tent " of amnion and a little air injected. The position of the bubbles tells at once whether the right cavity has been entered, and the inoculation is then completed. The egg is sealed with a coverslip and paraffin-vaseline as in the normal technique and re-incubated at 35° C. for three (sometimes two or four) days.

In examining the embryo after incubation we usually first remove some of the amniotic fluid aseptically with a Pasteur pipette, as this contains a high content of virus, and forms a suitable material for subinoculations. The embryo is removed to a sterile Petri dish and the contents of its trachea are removed with a suitably drawn-out capillary pipette. In infected embryos the fluid is turbid, in normal embryos perfectly clear, except for an occasional discrete flake of epithelium. Smears are prepared from this fluid and stained by Leishmans's method. If good cellular detail is required it is advisable to transfer the fluid to a capillary tube, seal one end and centrifuge. Films made from the deposit are usually very satisfactory.

The body cavity of the embryo is now opened and the lungs examined and removed. For histological work lungs were fixed in neutral formol saline and stained with haematoxylin and eosin.

Emulsions of tissue for subinoculation or titration were prepared by grinding the organs (two lungs or an approximately equal mass of other tissues to 1 c.c. of broth) with powdered quartz and broth. After centrifugation the supernatant fluid was used undiluted or at the appropriate dilution in saline containing 10 per cent. of normal horse serum. Titrations on the chorio-allantois or by intranasal inoculation in mice were done according to the conventional methods.

RESULTS.

General Character of the Lesions Produced.

When eggs are opened three days after amniotic inoculation with a typical influenza strain most of the embryos look slightly sick, their development being retarded and the circulation weak. Anaemia shown by the paler colour of the blood is sometimes a feature, especially in chicks that survive four days. A proportion of deaths occur, some apparently traumatic, but most appear to be due to specific infection. Owing to the very rapid post-mortem changes, it is usually impossible to be certain whether or not a dead embryo has been specifically infected, and except in cases where death has obviously occurred within a few hours no significance has been attached to dead embryos.

The state of the tracheal fluid is the best single index of infection. If this appears turbid to the naked eye, showing both granular clumps of cells and a

background opacity, one can be certain infection has occurred. In all normal embryos the fluid is clear, except for an occasional flake of epithelium. Occasionally haemorrhage into the amniotic cavity, either at the time of inoculation or on removing the embryo, will result in the recovery of red cells from the tracheal fluid. Stained smears, preferably made from centrifuged deposit of the tracheal fluid, show characteristically vacuolated epithelial cells, leucocytes, and what appear to be immature red cells with disintegrated nuclei. A detailed account of the cytological findings will be published later. It may be noted, however, that many smears show appearances in the cytoplasm of epithelial cells which we are disposed to interpret as micro-colonies of the virus.

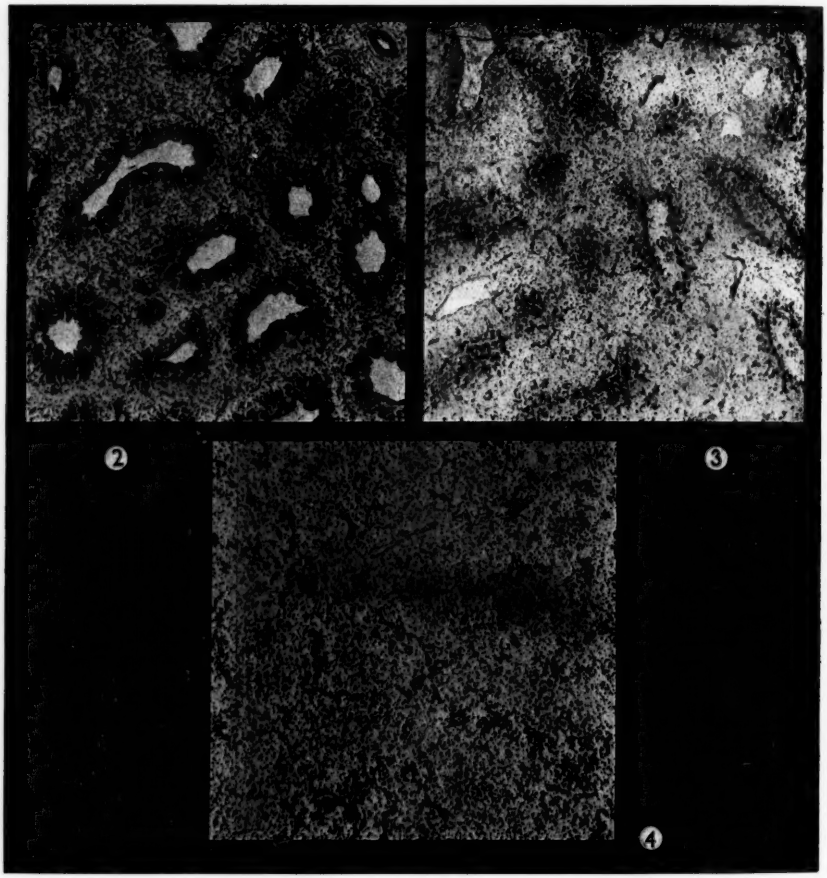
When the embryo is definitely sick, the appearance of the lung is characteristic. It is much whiter than normal, firmer, and lacks the usual surface pattern of more or less parallel lines. Many embryos with definite infection, indicated by positive tracheal fluid findings and subsequent histological section, show only very slight deviation from the normal appearance of the lungs. Gross macroscopic change appears to correspond with the final complete disintegration of internal structure seen in histological sections.

Histology.

In order to understand the histological changes it is necessary to give a brief description of the embryological development of the chick lung. The account which follows is derived from Lillie (1908) and Kerr (1919).

The lung develops from the ventral post-branchial region of the primitive pharynx. At 72 hours this is compressed to form the laryngotracheal groove, from the posterior end of which two buds develop to form the lung primorida. On each side a tubular bud grows backward with a mesenchymal sheath to form the extra-pulmonary bronchus and the mesobronchus (within the lung substance). From the mesobronchus there arise two sets of secondary bronchi, the entobronchi, which arise from the proximal part of the mesobronchus, and the ectobronchi, which arise more distally. The former are distributed on the whole ventro-mesially, the latter dorso-laterally. From both sets large numbers of tertiary tubules, the parabronchi, arise from the eighth to the thirteenth day. These grow in more or less parallel fashion toward one another, and about the thirteenth day those from the ectobronchi fuse with those from the entobronchi to form continuous tubes. At the same stage numerous outgrowths from the parabronchi appear disposed in radiating fashion which will eventually develop into the respiratory capillaries. It will be remembered that in the birds the gaseous exchanges take place not in blind alveoli, but in open tubules through which the air is drawn into and out of the various air sacs in the abdominal cavity and elsewhere.

At the stage of development in which we are interested, i.e. from the twelfth to the sixteenth day, the chief features of lung sections are the parabronchi embedded in loose mesenchymal tissue (Fig. 2). Depending on the plane of section, they appear either as circular or elongated slits lined with respiratory epithelium. In younger embryos they are simple, in older surrounded by very numerous saccular outgrowths. Each parabronchus lies in a roughly hexagonal area marked out by the developing blood vessels. Most



Figs. 2, 3 and 4 are photographs of sections stained with haematoxylin and eosin shown at a magnification of 75 diameters.

FIG. 2.—Normal chick embryo lung, 14 days.

FIG. 3.—Typical influenza virus lesion, showing desquamation of epithelium of parabronchi.

FIG. 4.—Final stage of lesion, showing complete disorganization of the lung. The roughly hexagonal areas corresponding to each parabronchus are vaguely outlined by strands of hypertrophic vascular endothelium.

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sections will also contain one or more of the larger bronchi, with a thicker layer of respiratory epithelium and no surrounding saccules.

The chief criteria of normality are (1) uniform and complete lining of the parabronchi with respiratory epithelium; (2) absence of degenerative change in the epithelial cells, apart from some goblet-cell appearances; (3) the lumina of the parabronchi are free from cells; (4) saccules, if present, are clearly outlined; (5) the mesenchymal tissue is composed of loosely packed uniform cells.

The earliest changes noted in influenzal infections take the form of disorganized areas of epithelium in some of the parabronchi. The cells are in irregular masses and show variable evidence of damage, most readily visible in haematoxylin-stained sections by changes in the distribution of nuclear chromatin. In the next stage the process has become general (Fig. 3). All the parabronchi show proliferation, disorganization and desquamation of their epithelial linings, and the lumen is more or less filled with damaged cells, amongst which disintegrated nuclei are conspicuous. The saccular outgrowths from the parabronchi soon lose their outline, and the whole lung becomes a disorganized mass of cells in which even the slits representing the parabronchi disappear. At this stage the roughly hexagonal areas corresponding to each parabronchus may be still clearly marked out by proliferation of the endothelium of the blood vessels at the margin of each area (Fig. 4).

The nature of the cells in the tracheal fluid is clearly related to the state of the parabronchi. In the early stages desquamated epithelial cells with specific cytoplasmic and nuclear vacuolation predominate, leucocytes appear later, and in smears from embryos surviving on the fourth day epithelial cells are almost absent, but leucocytes are very numerous. Cells which we consider to be immature red cells with disintegrated nuclei are commonly seen, both in sections and in smears, but their significance is not yet clear.

Propagation of Unadapted Virus Strains.

No opportunity has arisen of testing material direct from human cases of influenza, but we had available the glycerolated turbinates of a ferret inoculated with pooled throat washings from four typical cases in July, 1939. This was the original isolation of the strain " Bundoora " described by Burnet and Lush (1940). An emulsion was prepared from this seven-months-old material, and after centrifugation four eggs were inoculated amniotically with the supernatant fluid. Two opened at three days and two at four days gave the typical findings described above.

Titration of the virus content of amniotic fluid from one of the embryos opened at three days showed it to be active (by amniotic route) to a dilution of 10^{-4}. Lung emulsified in 1 c.c. of broth was active undiluted, but not at 1 : 100 or 1 : 1000.

Passage of this strain has continued to date (13 passages), the usual inoculum being pooled lung emulsion and amniotic fluid. Material from the fifth passage was inoculated intranasally in a ferret, which responded with a typical biphasic temperature reaction with the first peak in 48 hours and the usual symptoms.

Another recent ferret strain, "Reid," described by Burnet and Lush (1940), is also being maintained by amniotic passage. By the twelfth passage

this strain had developed a relatively high virulence for mice, four of eight inoculated dying within eight days.

The following representative mouse strains have also been shown to produce the characteristic changes in the chick embryo lung and tracheal fluid: "Melbourne," "W.S.," "Bur" (Smith and Andrewes, 1938), "Reid." A strain of swine influenza virus maintained in mice also produced lesions in no way different from those of the human types.

The reactions of well-adapted egg strains have not yet been properly investigated, but the results so far have been irregular, and suggest that much of the tissue specificity of the strains has been lost.

Failure of Other Viruses to Infect.

The specificity of the reaction for pneumotropic viruses is indicated by our failure to produce similar lesions with a variety of virus strains, most of which are capable of free growth on the chorio-allantois. Wholly negative results were obtained with herpes, louping ill, myxomatosis, fowl-pox and ectromelia viruses. Rather surprisingly the virus of infectious laryngotracheitis of fowls showed the same lack of effect, but the strain used was of low virulence, and the experiment requires to be repeated with a more active strain.

Psittacosis virus has given two positive results in the four lungs which have been sectioned, and further work with this virus is being undertaken.

DISCUSSION.

In this preliminary communication our intention is merely to provide a fairly detailed account of the technique and to indicate its potential usefulness as a method of virus research. On the theoretical side it provides a particularly interesting example of tissue specificity. It is a nice problem to determine what is the common factor which makes the developing epithelium of the chick's parabronchi and the respiratory epithelium of man and the ferret highly susceptible to influenza viruses, and why this susceptibility disappears in the grown fowl.

Amniotic inoculation is in a certain sense equivalent to placing the embryo in an infected external environment from which infection can be derived by more or less "natural" routes. It is possible that this circumstance could also be applied to the study of viruses other than pneumotropic ones.

From the practical side we feel that the technique is particularly applicable to the study of those influenza-like infections of the human respiratory tract which may be due to as yet undiscovered viruses. It is possible, of course, that the susceptibility of the chick lung is a mere lucky accident, and that only the influenza viruses can produce the lesions described, but until this is proved, it seems highly desirable that the method should be included in any research on infectious respiratory diseases of unknown aetiology. In the influenza virus field it may provide a useful means of primary isolation from human cases without recourse to the ferret. Another potentiality worth consideration is for the production of antigenic material from virus as little removed from the original human type as possible. In the event of a pandemic due to an antigenically new type of influenza virus, it might be of the greatest value to

be in a position to produce large quantities of virus in the universally available chick embryo immediately the responsible virus is isolated.

SUMMARY.

Influenza virus strains can be propagated in chick embryos without preliminary adaptation when the inoculation is made by the amniotic route. The technique of this method is described.

Characteristic histological lesions and changes in the cellular content of the tracheal fluid can be used as indices of infection.

All influenza virus strains, including first-passage ferret material, well-adapted mouse strains of several types and swine influenza virus are active.

With the exception of psittacosis virus, none of the other viruses tested by this method produced any lesions in the lungs.

The potential usefulness of the method in several fields of virus research is discussed.

REFERENCES.

BUDDINGH, G. J., AND POLK, A. C.—(1939) *J. exp. Med.*, **70**, 499.
BURNET, F. M.—(1936) *Sp. Rep. Ser. med. Res. Coun., Lond.*, No. 220.
Idem AND LUSH, D.—(1940) *Aust. J. exp. Biol. med. Sci.*, **18**, 49.
KERR, J. G.—(1919) 'Textbook of Embryology. Vol. II, Vertebrata.' London (Macmillan).
LILLIE, F. R.—(1908) 'The Development of the Chick.' New York (Henry Holt).
SMITH, W., AND ANDREWES, C. H.—(1938) *Brit. J. exp. Path.*, **19**, 293.
STUART-HARRIS, C. H., SMITH, W., and ANDREWES, C. H.—(1940) *Lancet*, i, 205.

THE APPLICATION OF THE SHARPLES CENTRIFUGE TO THE STUDY OF VIRUSES.

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WE have already shown (McIntosh, 1935; McIntosh and Selbie, 1937) that viruses and bacteriophages can be efficiently centrifuged by means of the spinning-top centrifuge built on the principle of Henriot and Huguenard (1927). However, the volume of suspension that can be dealt with in centrifuges built on that principle, even with the multiple large bucket type designed by Bauer and Pickels (1936), is limited. Centrifugalization by means of closed rotating hollow cylinders was introduced by Schlesinger (1936), but, again, the quantity of suspension that can be centrifuged in one run is limited to the capacity of the cylinder. We therefore decided to use a Sharples laboratory centrifuge with an open or separator type of cylinder, which gives a continuous flow through the cylinder and thus greatly increases the possibility of dealing with large quantities of suspension.

For the efficient centrifugalization of viruses and bacteriophages several modifications were necessary. In the first place the smallest delivery jet

supplied with the centrifuge does not allow a slow enough passage of virus suspensions through the cylinder for the sedimentation of these very small particles. If the rate of delivery of the suspension is cut down to the delivery required the jet of fluid is not high enough, as it must be at least 2 cm. in height to enter the cylinder, and leakage at the inlet occurs.

We therefore introduced smaller delivery jets, and found that with the slower rate of delivery a very large proportion of the particles were retained in the cylinder running at 40,000 r.p.m. A certain proportion of the particles could be collected from a cellophane liner, but the remainder were found to be suspended in the fluid which drained from the cylinder at the end of the run. In the ordinary type of separator cylinder the volume of fluid at the end of the run is over 200 c.c. and is too large for the efficient handling of virus suspensions. We therefore obtained a new type of separator cylinder made of well-tinned steel (Fig. 1, B), and introduced a duralumin tube within the cylinder so that the volume of fluid retained at the end of the run is now only 9 c.c.

We have also been able to counteract spraying from the delivery spouts and other disturbances caused by currents of air passing through the cylinder by fitting a stopper inside the head of the cylinder. A description of the modifications we have made and a short account of some of our results is given.

TECHNICAL.

Modifications in the Centrifuge.

Turbine nozzle.

A new air delivery nozzle to the turbine with an internal diameter of 2·5 mm. has been fitted because the capacity of our compression plant (McIntosh and Selbie, 1937) is not sufficient to maintain the pressure required (40 lb. per sq. in.) with the original wider nozzle. With the smaller nozzle it is possible to maintain constant pressures up to at least 40 lb. per sq. in., giving a speed of 40,000 r.p.m. and a gravimetric pull in the neighbourhood of 35,000 times gravity.

Delivery jets.

Smaller delivery jets were prepared by soldering short lengths of stainless steel hypodermic needle tubing into the original jets as shown in Fig. 1 (D). By using tubing of wire gauge Nos. 1 and 14 and varying the head from 15 to 48 in. any rate of delivery from 7 to 30 c.c. per minute can be obtained.

Measurement of speed.

A new turbine lid with a central window and a piece of stout celluloid of the same size underneath have been fitted so that the upper surface of the turbine wheel, on which has been painted a V-shaped mark, can be seen. It is thus possible to measure accurately the speed of the centrifuge by the stroboscope as we have already described (McIntosh and Selbie, 1937).

Modifications in the New Cylinder.

Duralumin liner.

When the centrifuge is running the new separator cylinder is lined by a layer of fluid 2·9 mm. in depth and 75 c.c. in volume. This fluid drains from the cylinder at the end of the run. A further reduction in the volume of the

residual fluid was obtained by introducing into the cylinder a closely-fitting duralumin tube (liner), the internal diameter of which is 4·75 mm. less than the internal diameter of the cylinder (Fig. 1, C). By this means the volume of the fluid was reduced to 13 c.c. and its depth to 0·55 mm.

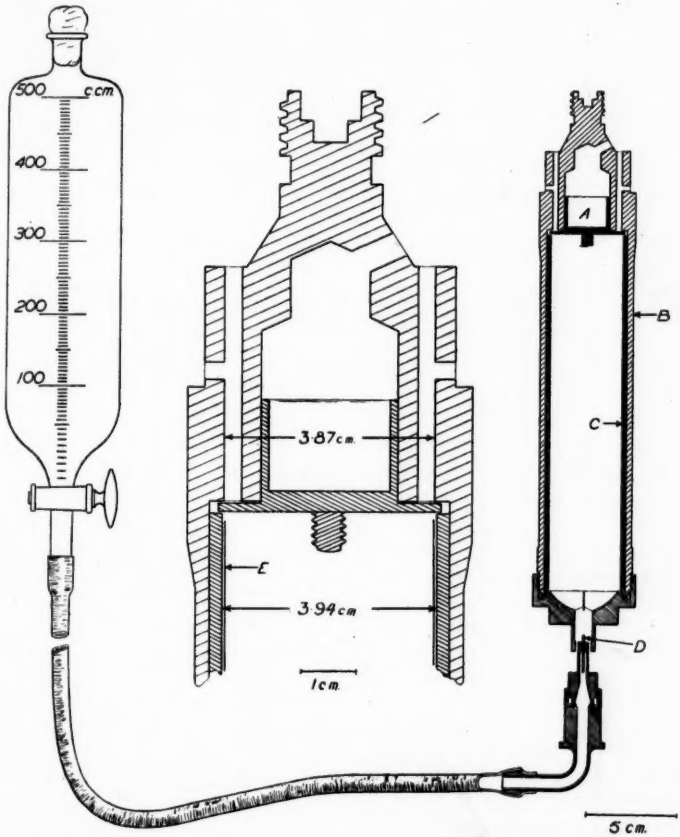

FIG. 1.—Delivery apparatus and new separator type of rotating hollow cylinder (B). The air stopper (A), the duralumin liner (C) and the new type of delivery jet (D) are shown in position. In the centre of the figure the head of the cylinder is shown on a larger scale, indicating the position of the cellophane liner (E). The diameters, 3·94 and 3·87 cm., show the outer and inner limits of the fluid layer when the cylinder is rotating at high speed.

Cellophane liner.

This is a sheet of cellophane, 0·16 mm. thick (No. 2400), used as a liner fitted inside the duralumin tube as shown in Fig. 1 (E). This further reduces the fluid layer to a volume of 9·0 c.c. and a depth of 0·37 mm. The cellophane is cut to the length of the tube and to a width 1·0 cm. more than the circumference of the tube so that when the cellophane is in position there is an overlap.

Care must be taken that the overlapping, inside, edge goes in a clockwise direction, otherwise the fluid will creep under and buckle the cellophane when the centrifuge is accelerating.

Stopper.

The makers of the centrifuge supplied a winged stopper, fitted into the body of the cylinder, but it could not be used with any form of liner. We therefore designed a stopper which is fitted into the head, but is free of the body of the cylinder. It consists of a disc 5 mm. less in diameter than the internal diameter of the cylinder, with a split collar to fit into the head of the cylinder above the outer effluents (Fig. 1, A). A short threaded peg is fitted to the centre of the lower surface of the disc so that the stopper can be removed for cleaning by a metal rod with a corresponding female thread.

Use of Modified Sharples Centrifuge.

Speed of centrifuge.

As with the spinning-top type of centrifuge, once the air-pressure required for a particular speed is determined with the stroboscope, all that is required to get the same speed again is to set the air-valves at the same pressure, e.g. 40 lb. per sq. in. air-pressure is required for a speed of 40,000 r.p.m.

Delivery of suspension.

The suspension is delivered to the centrifuge from a graduated separating funnel as shown in Fig. 1. As has already been mentioned, the rate of delivery can be regulated by changing the jet or altering the head. An alteration of the rate of delivery is equivalent to a change in the time of spinning, for as the rotating cylinder retains a constant volume of 9 c.c. of suspension during the run, the time of spinning is equal to the time in which 9 c.c. of suspension is delivered. In most of our experiments with viruses and bacteriophages we have used delivery-rates of from 15 to 20 c.c. per minute, so that the duration of spinning for these particles has been from 25 to 40 seconds.

The correct delivery-jet and head can usually be selected from previous experience, but if the suspension is more viscous than usual (e.g. filtrates of fowl tumours) the head may have to be considerably increased. The separating funnel is first filled with the suspension, and the cylinder stop-cock is then opened in order to fill up the rubber tubing connection to the delivery-jet, care being taken to avoid air-locks. When the centrifuge has attained uniform speed at the appropriate air-pressure, delivery is started by releasing a clip just proximal to the delivery-jet holder. The rate of delivery is checked from time to time to ensure that there is no interruption in the flow from blocking of the delivery-jet by small coagula which sometimes form in virus suspensions, particularly in fowl tumour filtrates. This can be avoided by running the suspension into the separating funnel through a sintered glass- or other coarse filter.

Collection of samples.

Supernatant fluid.—The supernatant fluid is collected in a large beaker or any wide-mouthed vessel placed at least 3 or 4 in. below the delivery spout. If it is placed too near the spout the current of air issuing from the spout

will be interfered with and cause spraying of the supernatant fluid. During the run no attention is required except to refill the separating cylinder from time to time, and to dispose of the supernatant fluid. When all the suspension has been run through it is followed by about 200 c.c. of saline or distilled water to displace the last traces of the suspending medium. This procedure also serves to remove some of the soluble material in the deposit.

Deposit.—At the end of the run the compressor is stopped, the delivery-jet is withdrawn from the cylinder, and the centrifuge is allowed to coast to a standstill. When the centrifuge has almost run down a Petri dish is placed under the cylinder to collect the fluid which still remains in the cylinder. About 7 c.c. of fluid drains out and a little more can be obtained by giving the cylinder a few gentle jerks. This residual fluid is retained for resuspending the deposit.

The cylinder is now removed and may feel slightly warm, but in our experience never reaches a temperature of more than 26° C. Sterile precautions must be observed in opening the cylinder and removing the cellophane, especially in experiments requiring subsequent bacteriological examination. If necessary, the cellophane is eased from the metal tube by inserting a long thin, sterile glass rod. The cellophane is then transferred to a 12-in. Petri dish, with the inner surface facing upwards. A little of the residual fluid is run over the surface, which is then scraped with a safety razor blade held vertically. The fluid with the suspended scrapings is collected to one side and pipetted off to a tube. This process is repeated several times until the deposit is completely removed.

Sterilization and Cleaning.

The cylinder with the duralumin and cellophane liners in position, the collecting covers and the delivery apparatus are all sterilized in the autoclave. In order to clean the cylinder at the end of the run the apparatus should be placed in 2 per cent. lysol for at least an hour. At the end of this time the apparatus should be cleaned in soapy water with the aid of a bottle brush, then well rinsed in water and dried thoroughly with a cloth.

Precautions.

Care should be taken to prevent over oiling, otherwise the oil creeps up the cylinder and contaminates the supernatant fluid. When infective suspensions are being dealt with, the supernatant fluid issuing from the spout is screened by cloths soaked in 2 per cent. lysol. It is always advisable to reduce dust particles in the centrifuging room by flushing the floor, for there is some circulation of air through the cylinder, even when it is supplied with a suitable stopper.

EXPERIMENTAL.

The aim of our experiments with the modified Sharples centrifuge has been to obtain concentrated suspensions of viruses and bacteriophages in as high a state of purity as possible. Apart from purification by the use of the centrifuge we have found that certain procedures in the preparation of the suspensions are of great value in attaining this end. It is desirable in the first

instance that the experimental material should be raised to the highest possible titre, and that, in the preparation of suspensions from animal tissues, only tissues of the highest infectivity should be chosen. Thus an initial advantage is gained in that the proportion of the infective particles to the contaminating tissue or medium is raised. The host proteins can be further reduced by digestion with trypsin—a procedure which we have used in preparing suspensions of Rous sarcoma virus. A high dilution of the suspension, giving a greater dispersion of the infective particles and of the less sedimentable extraneous material, is also of great value.

The method of centrifuging is also of great importance in obtaining pure preparations. The centrifugal force applied should be just sufficient to reduce the titre of the supernatant fluid to 1/10 of the original suspension. The number of the particles thus retained in the deposit would be 90 per cent. of the total number in the original suspension, and they would be free of a large amount of less sedimentable material which would have been brought down with a greater centrifugal force. As has been mentioned before, the suspending fluid in the cylinder at the end of the run can be completely displaced by saline or water, which also serves to remove some of the soluble contaminating material from the deposit. The washing or purification of the deposited particles can be continued by resuspending the deposit in a large volume of saline or water and again spinning in the Sharples centrifuge.

Some of our results are summarized in Table I. It will be noted that the supernatant fluid was reduced in infectivity at least 100 times in all experiments except with Rous sarcoma virus, which was reduced only 10 times. In considering the titre of the deposit it must be noted that the deposit is suspended in a volume of 7 to 8 c.c. If all the particles are recovered, the highest concentration possible is that represented by the proportion of the volume of the original suspension to the volume of the concentrate. Thus, if all the particles in an 8-litre suspension are recovered in 8 c.c., the suspension is concentrated 1000 times. The experiments with *Staphylococcus aureus* and *B. prodigiosus*, where comparatively accurate bacterial counts could be made, show that all the organisms can be recovered. The other experiments appear to show some variation in this respect, but all the results come within the expected variation when ten-fold dilutions are used for the titration of samples, and show that all the infective particles in the original suspension can be recovered in the deposit except for the small proportion which escapes in the supernatant fluid.

We have also estimated the purity of some of the concentrates by comparing the dry weight of the minimal infective dose and the weight of the infective particle, calculated from our previous findings (McIntosh and Selbie, 1937). The results are shown in Table II. On this estimation the concentrates of *Staphylococcus aureus* and *B. coli* bacteriophage would appear to consist entirely of active infective particles. On the other hand, the vaccinia and Rous sarcoma virus concentrates appear to consist mainly of extraneous material. This extraneous material most probably consists of sedimentable debris from the tissues of the host, for it has been shown in the experiment with *B. coli* bacteriophage, which is free from such contaminants, that the total deposit can be accounted for as active particles. In any case, judging

TABLE I.

Suspension.	Volume. (c.c.)	Rate of delivery. (c.c./min.)	Speed. (r.p.m.)	Titre. Original.	Titre. Supernatant.	Titre. Deposit.
Staphylococcus aureus	1000	650	21,000	$1{\cdot}3 \times 10^7$	$1{\cdot}1 \times 10^5$	$1{\cdot}3 \times 10^9$
B. prodigiosus	1000	450	21,000	$1{\cdot}5 \times 10^7$	$7{\cdot}3 \times 10^5$	$3{\cdot}7 \times 10^9$
Pleuropneumonia	1000	80	21,000	10^7	10^5	10^{10}
Vaccinia	1500	20	40,000	10^3	*nil*	10^6
Rous sarcoma virus	4000	20	40,000	10^2	10^1	10^4
Staphylococcus bacteriophage	1000	18	40,000	10^4	10^1	10^6
B. coli bacteriophage	9000	16	40,000	10^{10}	10^5	10^{14}

TABLE II.—*Comparison of Minimal Infective Dose of Deposit and Theoretical Weight of Particle.*

	Total dry weight of deposit. (mg.)	Titre of deposit.	Minimal infective dose of deposit. (mg.)	Weight of particle. (mg.)
Staphylococcus aureus	465	3×10^{12}	$1{\cdot}6 \times 10^{-10}$	$3{\cdot}4 \times 10^{-10}$
Vaccinia virus	9·4	10^6	$3{\cdot}4 \times 10^{-7}$	$5{\cdot}2 \times 10^{-12}$
Rous sarcoma virus	14·2	10^4	$5{\cdot}1 \times 10^{-5}$	$2{\cdot}9 \times 10^{-13}$
B. coli bacteriophage	26·9	10^{14}	$9{\cdot}5 \times 10^{-14}$	$6{\cdot}0 \times 10^{-14}$

TABLE III.—*Chemical Composition of Deposits.*

	Minimal infective dose. (mg.)	Extractable lipoid. (per cent.)	Non-lipoid fraction (per cent.). Nitrogen.	Carbohydrate (as glucose).	Ash.	Total phosphorus.
Staphylococcus aureus	10^{-10}	0·6	12·3	7·9	8·2	2·6
B. coli	10^{-10}	1·6	13·6	15·0	5·1	1·9
Vaccinia	10^{-7}	46·0	15·0	9·6	5·0	1·3
Rous sarcoma No. 1	10^{-5}	39·0	13·3	8·7	2·0	1·03
Tumour 7*	..	42·0	13·3	15·7	3·0	1·15
Sheffield dibenzanthracene tumour†	..	39·0	12·7	8·2	0·7	1·1
B. coli bacteriophage	10^{-13}	10·4	13·4	25·0	6·0	2·9

* Non-filterable fowl tumour (McIntosh and Selbie, 1939).
† Non-filterable fowl tumour (Mellanby, 1934).

from the titres of the original suspensions of vaccinia and Rous sarcoma and assuming that an infective dose is represented by one particle, the total weight of active virus would be about 10^{-4} mg. in the vaccinia suspension and about 10^{-7} mg. in the Rous sarcoma suspension. It is therefore clear that weighable amounts of virus cannot be prepared unless titres of about 10^{10} are obtained in the original suspension. Weighable amounts can also be obtained by the use of very large quantities of material as in plant virus work, but this method is impracticable with animal viruses.

DISCUSSION.

Our results show that by means of the modified Sharples centrifuge virus or bacteriophage can be efficiently sedimented from a large quantity of suspension and concentrated in a small volume, with complete removal of the original suspending medium and without loss of activity. As judged by a comparison of dry weight and activity, a concentrate of *B. coli* bacteriophage is the only one of our preparations of virus or bacteriophage that has so far shown a high degree of purity. The chemical analyses of some of these concentrates have been published by Pollard (1939) and are reproduced here (Table III). The *B. coli* bacteriophage preparation contained a high proportion of nucleic acid, which would further indicate that this preparation contained a high proportion of bacteriophage particles. The analyses of the Rous sarcoma and vaccinia preparations were also of interest in that they showed a nitrogen content similar to the bacteriophage preparation and a high percentage of fat, as has been found by other workers. The high fat content of the vaccinia preparation conforms with the conception that this virus is associated with a fatty envelope (McFarlane, Macfarlane, Amies and Eagles, 1939).

SUMMARY.

Several modifications are described by which the Sharples laboratory centrifuge has been adapted for the efficient sedimentation and concentration of virus or bacteriophage from large quantities of suspension.

Preliminary results show that concentrates of high purity can be obtained by this method without loss of activity.

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REFERENCES.

BAUER, J. H., AND PICKELS, E. G.—(1936) *J. exp. Med.*, **64**, 503.
HENRIOT, E., AND HUGUENARD, E.—(1927) *J. Phys. Radium*, **8**, 433.
MCFARLANE, A. S., MACFARLANE, M. G., AMIES, C. R., AND EAGLES, G. H.—(1939) *Brit. J. exp. Path.*, **20**, 485.
MCINTOSH, J.—(1935) *J. Path. Bact.*, **41**, 215.
Idem AND SELBIE, F. R.—(1937) *Brit. J. exp. Path.*, **18**, 162.—(1939) *Ibid.*, **20**, 49.
MELLANBY, E.—(1934) *Ann. Rep. Brit. Emp. Cancer Campgn.*, **11**, 81.
POLLARD, A.—(1939) *Brit. J. exp. Path.*, **20**, 429.
SCHLESINGER, M.—(1936) *Nature*, **138**, 549.